Acquisition Directorate

Research & Development Center

Report No: CG-D-13-13

Revised Protocol for Zooplankton Automated Analysis

November 2011

Homeland Security

N O T I C E

This document is disseminated under the sponsorship of the Department of Homeland Security in the interest of information exchange. The United States Government assumes no liability for its contents or use thereof.

The United States Government does not endorse products or manufacturers. Trade or manufacturers' names appear herein solely because they are considered essential to the object of this report.

This report does not constitute a standard, specification, or regulation.

Timothy R. Girton
Technical Director
United States Coast Guard
Research & Development Center
1 Chelsea Street
New London, CT 06320

Technical Report Documentation Page

1. Report No	2. Government Accession Number	3. Recipient's Catalog No.
CG-D-13-13		

4. Title and Subtitle	5. Report Date
Revised Protocol for Zooplankton Automated Analysis	November 2011
	6. Performing Organization Code Project No. 4101
7. Author(s) Nelson, Bruce; First, Matthew; Steinberg, Mia; Riley, Scott; Robbins-Wamsley, Stephanie; Herring, Penny and Drake, Lisa	8. Performing Report No. UDI # 1157
9. Performing Organization Name and Address Naval Research Laboratory Center for Corrosion Science Bldg F14, Trumbo Annex Key West, FL 33040 U.S. Coast Guard Research and Development Center 1 Chelsea Street New London, CT 06320	10. Work Unit No. (TRAIS) 11. Contract or Grant No. MIPR HSCG32-10-X-R00014
12. Sponsoring Organization Name and Address U.S. Department of Homeland Security U.S. Coast Guard Office of Operating & Environmental Standards Washington, DC 20593-0001	13. Type of Report & Period Covered Final 14. Sponsoring Agency Code Commandant (CG-5224) USCG Headquarters Washington, DC 20593-0001

15. Supplementary Notes

The R&D Center's technical point of contact is Penny Herring, 860-271-2868, email: penny.r.herring@uscg.mil

16. Abstract (MAXIMUM 200 WORDS)

Ballast water management systems must be stringently tested relative to the Coast Guard's proposed ballast water discharge standard for live organisms prior to Coast Guard approval for routine shipboard use. Analysis for live organisms in sparsely populated samples is time-limited and labor intensive. Skilled staff must use a microscope to observe, count, and verify viability of live organisms ≥50 µm in size. An automated method was sought to significantly reduce staff time and effort, maintain consistency, and provide an archive of analytical results. Commercially available laboratory equipment and computer software were used in conjunction with specially developed pattern recognition software. This report provides a description of the equipment required and the protocol developed. A step-by-step protocol is also provided.

The findings in this report are the result of continued research into the potential to automate zooplankton analyses and provide the most current information available at the conclusion of this initial research effort. Additional automation research efforts are ongoing and may affect details reported herein.

17. Key Words	18. Distribution Statement
Automated analysis, zooplankton, motion detection, ballast water treatment, pattern recognition, vital stains, epifluorescence	Distribution Statement A: Approved for public release; distribution is unlimited.

19. Security Class (This Report)	20. Security Class (This Page)	21. No of Pages	22. Price
UNCLASSIFIED	UNCLASSIFIED	48	

(This page intentionally left blank.)

EXECUTIVE SUMMARY

Ballast water is a known pathway for the introduction of aquatic nuisance species (ANS). In an effort to reduce the number of introductions of ANS into United States waters, the U. S. Coast Guard (USCG) has proposed regulations requiring ships to meet stringent discharge standards. The proposed concentration for organisms ≥ 50 microns µm (nominally zooplankton) is 10 living organisms per cubic meter of ballast water. Before the Coast Guard can approve ballast water management systems (BWMSs) for routine use aboard ships, the BWMS vendors must demonstrate they are capable of meeting the discharge standard.

A protocol for testing ballast water management systems at full scale has been developed by the Environmental Protection Agency's Environmental Technology Verification Program in cooperation with the USCG. Among other metrics, testing requires evaluating treated samples to determine the number of living organisms ≥ 50 µm. Current manual methods require skilled personnel using microscopes to observe, enumerate, and determine viability of organisms in concentrated samples before mortality occurs from the artificial conditions of holding samples (determined to be six hours, in one location). This visual analysis is labor intensive, requires skilled personnel, is subject to operator fatigue, and provides no archive of results. The U.S. Coast Guard Research and Development Center therefore sought a method to automate analyses of the ≥50 µm size class.

Initial efforts by researchers at the Naval Research Laboratory in Key West, FL had shown that pattern recognition algorithms could be applied to sequential photomicrographs of treated samples to identify motion, and therefore viability, of organisms. Subsequent work refining algorithms, improving equipment, and investigating appropriate vital and mortal stains led to a practical protocol that could be used routinely during tests of BWMSs.

This report provides background information on how the automation protocol was developed, describes the type of equipment used, and presents the protocol for use by other test facility operators. The text discusses setting up data archives and image collection. The basic protocol is discussed in the text, and a step-by-step protocol for routine laboratory use is provided in an appendix.

This research effort will provide a means to provide consistent analyses of organisms ≥ 50 µm in treated ballast water samples with significantly reduced, skilled staff-hours. The government-developed protocol will be available free of charge in the public domain.

The findings in this report are the result of continued research into the potential to automate analyses of organisms ≥ 50 µm and provide the most current information available at the conclusion of this research effort. The emphasis of this year's effort was processing the types of samples that would be encountered during the standardized testing of ballast water treatment equipment. Additional automation research efforts are ongoing and may affect details reported herein.

(This page intentionally left blank.)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS, ABBREVIATIONS, AND SYMBOLS

ANS	Aquatic nuisance species
BWMS	Ballast water management system
CMFDA	5-Chloromethylfluorescein diacetate
CCD	Charged coupled device
DIA	Diascopic (brightfield)
EPA	Environmental Protection Agency
FDA	Fluorescein diacetate
GFP	Green fluorescent protein
GUI	Graphical user interface
m^3	Cubic meter
mL	Milliliter
mm	Millimeter
ms	Millisecond
ND	Neutral density
NRL	Naval Research Laboratory
PC	Personal computer
RGB	Red green blue
S	Second
USCG	United States Coast Guard
X	Magnification power
µm	Micrometer (micron)
<	Less than
>	Greater than
≥	Greater than or equal to
.nd2	Image file type
.xls and .xlsx	Excel Spreadsheet file type

(This page intentionally left blank.)

1 BACKGROUND AND INTRODUCTION

Testing to evaluate the efficacy of a Ballast Water Management System (BWMS) requires the characterization of samples to determine the number of live organisms after treatment. Standardized BWMS testing requires that organisms be characterized in three size classes based on maximum dimension on the smallest axis: organisms ≥ 50 microns (µm) (nominally zooplankton), organisms ≥ 10 µm to < 50 µm (nominally protists), and organisms < 10 µm (here, aerobic, heterotrophic bacteria) (United States Environmental Protection Agency (EPA) 2010). The focus of this document is to provide protocols for collection of image sets, test documentation, image set analysis, and data archive for organisms in the ≥ 50 µm size class. The document also describes the equipment and software required to implement these protocols.

With respect to organisms in the ≥ 50 µm size class, Phase I of the U.S. Coast Guard's proposed discharge standard requires that there be less than 10 living organisms per cubic meter (m^{-3}) (U.S. Federal Register 2009). It is not feasible to reliably characterize a sparse assemblage of organisms in this large volume of fluid by direct observation. Consequently, statistical arguments require samples to be concentrated (up to 60,000:1) prior to optical evaluation (usually by microscope) when determining the efficacy of a BWMS. As standardized tests also require that suspended solids and other water properties (e.g., mineral matter and particulate organic carbon) fall within specified ranges, concentrating samples also increases the amount of suspended materials, making these samples significantly more complex to analyze since organisms can be obscured from view by sample debris.

The Naval Research Laboratory (NRL) Center for Corrosion Science and Engineering in Key West, FL has recently determined that the presence of this and other types of debris (e.g., algal cells) common to BWMS test samples makes it noticeably more difficult to manually count and characterize simple, regular objects (such as 50 µm – 150 µm size polymer microbeads) in test samples using manual microscopy methods, compared to enumerating these same objects in clean laboratory water. In addition, the complexity and diversity of organisms in the ≥ 50 µm size class make it challenging to accurately characterize these samples using manual microscopy methods. Furthermore, samples must be analyzed before samples degrade (i.e., organisms die off due to holding conditions), which is within six hours of collection at the NRL facility.

The motility of organisms in the ≥ 50 µm class presents another challenge, as some of these organisms can move rapidly across a typical sample well or counting chamber in a fraction of a second. Further, suspended solids in the sample can allow these organisms to "hide" or be obscured by debris during observation. The motility of these organisms necessitates the microscope observer view the entire sample well in a single view when conducting analyses or to track and account for these organisms as they enter and exit the field of view. The alternative approach, magnifying selected regions of the field of view, provides information regarding only organisms in the magnified region (which varies as a result of organism motility during the time of observation).

The requirement to observe the entire sample well can be addressed using a microscope and camera system combination with sufficient spatial resolution. This arrangement can be used to collect a time series of images of the sample well. When these systems provide adequate spatial resolution, it becomes feasible to "zoom in" on the individual organisms in the digital image set after the data has been collected from the entire sample well.

NRL has developed and demonstrated the utility of algorithms that use motility to classify the viability of organisms in the ≥ 50 µm size class (Lemieux, et al. 2007). This work focused on standard test organisms, including brine shrimp (*Artemia franciscana*) and rotifers (*Brachionus plicatilis* and *B. calyciflorus*). Initial work was conducted with homogeneous monocultures with little to no added debris in the samples. Towards the end of initial feasibility demonstrations, NRL used these algorithms to work with more complex samples and organism assemblages. This work clearly demonstrated the potential for using motility algorithms to assess viability in complex samples using light microscopy. This work also demonstrated that the microscope and camera systems available at NRL provided sufficient spatial resolution to observe individual organisms (and organism details) in digital image sets in which complete sample wells had been imaged.

NRL has also explored a variety of biological vital and mortal stains to determine the viability of protists. Recent work has focused on performing measurements at a variety of geographic locations to demonstrate that these stains provide a location-independent means to identify viable protists in test samples (Steinberg et al. 2011b). NRL recommends staining samples with a combination of two vital stains: Fluorescein Diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA, CellTracker™ Green) (Steinberg et al. 2011a). After entering living cells, non-specific esterases in the cell react with these compounds, resulting in a molecule that fluoresces green when excited with blue light. Work performed at NRL indicates that by using a combination of these two stains, the viability of a greater number of organisms can be determined (compared to using these stains individually). This work also has demonstrated that many organisms' fluorescence signals can be observed even when an organism is obscured by debris. The image sets produced using this microscope modality are extremely useful for identifying viable zooplankton in complex samples when these data are analyzed in conjunction with motility algorithms.

This year's effort focused on working with samples that are representative of those that are encountered during BWMS evaluations. This has included samples that are representative of those associated with tank filling operations, control tank drain operations, and test tank drain operations. Note that samples cover a wide range of organism concentrations. Significant effort has been made in establishing the properties of typical samples from each of these types of operations and in generating and analyzing representative samples.

2 APPROACH

Under this program, NRL developed protocols for the collection, documentation, analysis, and archiving of image data to support BWMS evaluations. This report will first describe the equipment and software required to collect data according to these protocols, followed by descriptions of the laboratory procedures used to collect and analyze image data according to these protocols. If other laboratories involved with BWMS testing use the equipment and software specified in this report, then the protocol described here should find broad application in supporting standardized testing of BWMSs.

It is assumed that samples have been prepared prior to the implementation of this protocol. For organisms ≥ 50 µm, this requires that samples first be concentrated to ensure that a sufficient sample volume is analyzed to achieve accurate analyses of a sparse population of zooplankton (< 10 viable organisms m^{-3}). The concentration factor will vary based upon sample quality and the type of BWMS evaluation operation that produced the samples. As an example, tank fill samples with their higher populations of viable organisms ≥ 50 µm may be concentrated to a value of 300:1 (sample volume: concentrate volume), while test tank samples with their sparse populations of viable organisms ≥ 50 µm may require the samples to be

concentrated by as much as 60,000:1. However, even in the case of test tank samples, the high concentrations of particles and aggregates may require a lower concentration factor to prevent debris in the sample from interfering with organism detection. This in turn would increase the number of sub-samples that would need to be analyzed to accurately characterize a sparse population of organisms.

Following sample concentration, the sub-samples are stained using a combination of CMFDA and FDA (Nelson et al. 2010a; Nelson et al. 2010b; Steinberg et al., 2011a, b). This staining procedure requires a ten-minute incubation period prior to sample analysis. Following staining and incubation, image data can be collected for a maximum of 20 minutes. Accordingly, the protocol allows images to be collected from a full sample well plate (24 wells) prior to exceeding this 20-minute period. It should be noted that NRL continues to perform work to determine if this 20-minute period can be extended.

Based on previous work, all sample concentration, staining, and image collection must be completed within 6 hours after obtaining a sample. Image and data analyses can be performed after this time, as they are performed on archived image sets that are collected according to this protocol.

2.1 Equipment

To characterize organisms in the ≥ 50 µm size range with this protocol, it is necessary to use two microscope modalities (i.e., brightfield [white light] and epifluorescence measurements) and to apply motility algorithms to both image sets. Combining these measurements allows the user to assess the viability of a wide range of organisms in the complex samples associated with BWMS evaluations. It is also necessary to be able to spatially correlate the image data from both of these modalities. Therefore, image sets from brightfield and epifluorescence must be interleaved, not collected in series.

In support of this program, NRL made additional modifications to its microscope and camera system to simultaneously collect both brightfield and epifluorescence time series image sets reliably with required image update rates. This was accomplished using a new light source and mechanical shutter that results in the microscope switching between brightfield and epifluorescent measurement modes under computer control with required timing precision and reliability. In this fashion, this equipment has been used to collect spatially correlated brightfield and epifluorescent image sets. For the analysis of zooplankton samples, 64 images are collected (32 brightfield and 32 epifluorescent). To eliminate problems that frequently occur with the first images from each microscope modality, they are deleted, resulting in the collected image sets consisting of 31 brightfield and 31 epifluorescent images that were collected over a 31-second observation window for each sample well.

2.2 Data

Data management and the documentation of system settings are critically important and assure the proper collection of image sets and archiving of test results. The imaging system (which includes the microscope, the camera, and the attached computer) used at NRL stores all of the image data from a given measurement into a single, large file (denoted as an *.nd2 file). Also, a spreadsheet (with embedded macros and forms) is used by the microscope operator to enter and record information specific to a given test. The operator also fills out specific fields in this spreadsheet to document all of the system settings. The spreadsheet and its embedded forms also provide a checklist to ensure that the operator has made all necessary system adjustments prior to data collection. This test specific Excel spreadsheet maintains this information in an .xls or .xlsx file. Much of these same data are also stored as metadata in the test-specific *.nd2 file.

Both of these files have the same basic name, with two exceptions: 1. the application extension, and 2. a file name suffix with three digits (e.g., 001) to count replicate measurements from a single sample. The three-digit counter indicates that the files are collected and analyzed with the same settings. Consequently, all data associated with a given test are stored in two files that have very similar names but different application extensions (i.e., .xls and .nd2). These files are named in a manner that allows many of the test details (e.g., test date, type of sample analyzed, some system settings, and replicate or sub-sample number) to be discerned from just the file name. This procedure facilitates file recognition and retrieval when reviewing multiple tests.

Data are archived by storing files in a directory with an intuitive structure. First, the directory hierarchy separates data by the year, month, and day of image set collection (each is a separate directory – e.g., C:\2011\03_Mar\15). The Excel spreadsheets described above are stored directly into a specific test directory under the day directory. Each *.nd2 file is stored in its own directory under the specific test directory. This directory has the same name as the *.nd2 file without the application extension (described in more detail in Appendix A). The file naming and directory structure provides a means to easily access data associated with a given test. Further, the file-naming conventions allow important information about the test to be discerned prior to analyzing the data. Lastly, the approach used by NRL requires only two files (both automatically generated during the data collection process) to archive all settings, other relevant test information, and the test data (image sets) itself associated with a given microscope measurement. Many of the systems settings are redundantly archived in both of these files, providing additional corroboration of proper system settings.

Data analysis is performed using a stand-alone application developed by NRL. When launched, this application provides a graphical user interface (GUI) that asks the operator to specify the *.nd2 file to be analyzed. The analysis application parses the *.nd2 file, performs all analyses, and stores its results in the same Excel spreadsheet that was used to document test-specific settings. The current focus of these algorithms is to enumerate the number of viable organisms in a test sample. The prototype analysis algorithms developed in previous efforts counted all observed organisms ≥ 50 µm and determined their viability (by detecting movement). The change to enumerating only *living* organisms ≥ 50 µm was implemented for two reasons: first, the number of *living* organisms is specified in discharge standards, and second, the increased complexity of BWMS samples rendered it too difficult to enumerate all organisms ≥ 50 µm.

3 EQUIPMENT AND SOFTWARE REQUIREMENTS

The imaging system, consisting of the microscope, camera, and control hardware and software (e.g., a personal computer [PC]), discussed in this section of the report is that used at NRL. The capabilities that are provided by this system are required for the proper implementation of this protocol. The overall requirement for system resolution for this equipment is to provide at least 10 µm resolution while imaging a full sample well of approximately 16 millimeters (mm) in diameter. The well should accommodate a sample volume of between 0.5 and 1.5 milliliters (mL). Such a requirement is to maintain a water column consistent with a microscope's depth of focus when imaging organisms in the ≥ 50 µm size class. The microscope system must also be capable of switching automatically between brightfield and epifluorescence modalities under computer control. This switching must be accomplished rapidly enough to afford a 1-second interval between successive images that comprise the image set.

The U.S. Coast Guard neither endorses nor recommends specific equipment or manufacturers. The concepts and algorithms developed in this project were developed for the specific microscope and camera systems resident at NRL. Other equipment with equivalent properties and attributes that provide the resolution and capabilities necessary to support this protocol can be used. However, many key system parameters that are currently monitored by system software may not be recorded as image file metadata with different hardware and operating software. Further, analysis routines will need to be modified to parse image data from a different source and may need additional modification if camera resolution and dynamic range are not identical to those used in the NRL systems.

3.1 Sample Well Plates and Unruled Sedgwick Rafter Counting Chambers

The resolution requirements for the microscope and camera systems are driven largely by the area imaged regardless of the specific method that is used to hold sample. The overall requirement is to collect images that provide < 10 µm spatial resolution when zoomed in. In the earlier protocol, a second requirement to observe the entire sample well was additionally put forward (Nelson et al. 2010); however, that requirement seems no longer necessary (see below).

NRL performed a series of evaluations on sample well plates. Two major criteria were used: First, it was important that debris in the sample well remain evenly dispersed throughout the well and not move during the observation. In many of the well plates evaluated, the combination of well shape and materials resulted in debris migrating to the center of the sample well during the observation window (30 seconds). This movement is important, as debris motion can be confused with organism movement by motility algorithms. Second, well plates needed to be constructed of scratch-resistant materials. Based on these criteria, NRL used the SensoPlate™ Glass Bottom Cell Culture Plates (Item # 692892; Greiner Bio-One, Monroe, NC) for analyzing zooplankton samples in the ≥ 50 µm size range. This well plate contains 24 wells, approximately 16 mm in diameter and suitable for containing 0.5 mL to 1.5 ml of fluid. NRL limited the sample volume to 0.5 mL to maintain sample depths consistent with typical microscope depth-of-focus specifications.

Figure 1. Photograph of the SensoPlate™ Glass Bottom Cell Culture Plate.

When imaging complex samples in the ≥ 50 µm size class, two problems were encountered with the sample wells. First, because of microscope alignment issues, portions of the sample wells were occluded. This was only observed in the brightfield illumination modality. When it occurs, this occlusion can prevent organisms at the edge of the wells from being observed using this microscope modality. Second, reflections of stained organisms located at the well perimeter can create multiple signals for even single stained organisms. This is only an issue for the epifluorescent microscope modality. These reflections complicate counting organisms under epifluorescent illumination.

Work performed using unruled, 1-mL Sedgwick Rafter counting chambers in support of the Protocol for Automated Protist Analysis (Nelson et al in preparation) had shown that these types of problems do not occur. Accordingly, this same approach was used for samples in the ≥ 50 µm size range.

When used to analyze samples in the ≥ 50 µm size class, the microscope zoom is adjusted such that half of the counting chamber, equivalent to 0.5 mL sample volume, is observed. This affords a setup without occlusions or well edge reflections and provides the required < 10 µm spatial resolution. Further, during "production analyses", both halves of the chamber can be analyzed independently and multiple chambers can be simultaneously positioned under the microscope. The microscope's automated X-Y stage can then be used to analyze multiple samples in a similar fashion to a multi-well tray. The only disadvantage of this approach is that organisms can swim out of the field of view during the observation window. The impact of this is mitigated to some extent because the organism can be dynamically observed as it swims out of or into the field of view.

Appendix B of this report provides a more detailed discussion of methods to hold samples in the ≥ 50 µm size class and how this affects the quality of data.

3.2 Microscope

NRL worked with Nikon to design a microscope with the desired capabilities for use with a Q-Imaging 2000R Retiga IEEE 1394 camera system. The desired capabilities include:

- Ability to image a 16 mm – 20 mm diameter field of view with < 10 µm spatial resolution
- Capability to collect images using brightfield illumination and epifluorescence
- Ability to rapidly switch between brightfield and epifluorescent modes under computer control
- Ability to move the sample well tray or counting chamber under computer control so all sample wells on a tray or appropriate portions of a chamber can be imaged
- Ability to focus the microscope under computer control
- Ability to monitor key system settings under computer control

3.2.1 NRL Microscope

NRL used the Nikon Multizoom AZ100 Multi-Purpose Zoom Microscope. As purchased, this microscope provides basic magnification of 5X, 10X, 20X and 50X with an 8:1 variable zoom available at each magnification. Implementation of this protocol does not require the four basic magnifications described above (Note the total magnification above is a combination of the objective magnification (variable) and the ocular magnification (10X)). To support work with sample well trays using the protocol provided in this report, the microscope is always operated at a basic magnification of 5X with the variable zoom set to 1.85X; to support work with Sedgwick Rafter counting chambers the same microscope magnification

settings are utilized. Consequently, a single objective is used to support these protocols. This microscope was originally purchased with the Nikon AZ-FL epifluorescence attachment, which allows the microscope to operate in both brightfield and epifluorescence modes. The approximate cost of the microscope with the epifluorescence attachment (and all required peripherals) was $30,000.

3.2.2 Light Sources

In December 2010, Nikon integrated a new epifluorescence light source into the imaging system (Lumen Pro™ Fluorescence Illumination System [Prior Scientific, Rockland, MA] equipped with a 200 W metal arc lamp). This additional light source allowed faster and more reliable switching between brightfield and epifluorescence light sources through the software interface. The cost of these light sources and the appropriate control software was approximately $10,000. This light source replaced a computer controlled light source that was installed by Nikon in July 2009. Work performed at NRL indicated that the first computer controlled light source did not provide the reliability and timing precision required for this application.

3.2.3 Sample Stage

In October 2009, Nikon integrated a computer controllable, motorized X-Y-Z stage to the NRL microscope. This stage allows each sample well on the sample well plates to be moved into position (and image sets collected) under computer control. The stage also provides the capability to focus the microscope under computer control. The cost of this motorized stage was approximately $15,000.

3.3 Imaging and Image Acquisition Software

Nikon NIS-Elements Advanced Research Imaging System Software (Elements) is used to control the microscope system during automated data collection. This software controls the entire image acquisition process, the storing of image data into a single *.nd2 file, the switching of the light sources during image acquisition, and the movement of the motorized stage when multiple sample wells are evaluated in sequence on a single multi-well plate. The software also controls the camera settings and provides image display and image processing capabilities. The cost of this software package was approximately $ 3,600.

3.4 Camera

NRL uses a Q-Imaging 2000 R Retiga IEEE 1394 camera, which provides 1600 x 1200 pixel resolution using a large area charged coupled device (CCD) detector that provides high light sensitivity (compared with consumer CCD-based imaging systems, such as video cameras). The camera is fully controllable using the NIS-Elements software. For this application, the monochrome, uncooled version of the camera is recommended. The cost of this camera is approximately $7,000. An even higher resolution camera (that will result in still more improved spatial resolution) that uses a 2048 x 2048 pixel detector is available at a cost of approximately $ 9,000. Both cameras are fully compatible with the Nikon Elements Software.

3.5 Computer

To complete the system, a PC with at least two RS-232 ports, four USB-2 ports, and an IEEE 1394 (Firewire) interface is required. The cost of an appropriate PC and display for the imaging system is estimated at $ 3,000.

3.6 Settings

Following the October 2009 and December 2010 updates to the microscope system, the majority of system settings are either directly monitored by the computer or entered by the microscope operator into the spreadsheet (as discussed in the next section). Many of the parameters entered into the spreadsheet can be corroborated, as these same data are recorded in the *.nd2 files. There are currently four parameters that are adjusted manually by the microscope operator that cannot be corroborated in the *.nd2 files. First, the operator manually adjusts (or ensures) that the variable zoom level is set to 1.85X for implementation of the data collection protocol. The operator is asked by the spreadsheet to confirm this zoom level prior to the start of data acquisition. Second, the filter cube position is manually set to position 2 – for the Green Fluorescent Protein (GFP) filter cube. Again, the operator is asked by the spreadsheet to confirm the filter cube position prior to the start of data acquisition. Third, the microscope operator needs to ensure that the 0.5X objective (5X total system magnification with the ocular lenses) is used. The operator is also asked to corroborate this point prior to data acquisition. Lastly, the microscope operator needs to ensure that the neutral density (ND) filters are in the proper configuration: the ND2 filter should be not engaged and the ND8 and ND16 filters should be engaged. The microscope operator is asked to corroborate this configuration prior to data collection.

3.7 Data Analysis Software

A series of algorithms was developed by NRL to analyze the images collected and stored by the Elements software. The algorithms analyze the images in the selected file and store the results in the file's associated spreadsheet file. This government-developed software for analyzing the collected data will be made available at no cost

3.8 Total System Cost

The total cost of a new system for implementing the protocols described in the next section of this report is approximately $69,000 (Table 1). With the upgrades made in its microscope system, the imaging system at NRL is now also configured to implement the protocols provided in the next section of this report.

Table 1. Equipment summary and costs.

Item	Model Used	Approximate Cost
Microscope	Nikon Multizoom AZ100 Multi-Purpose Zoom	$30,000
Light Source	Nikon Computer-Controlled Light Switcher	$10,000
Sample Stage	Nikon Motorized XYZ Stage	$15,000
Imaging Software	Nikon NIS-Elements Advanced Research Imaging System Software	$3,600
Camera	Q-Imaging 2000 Retiga IEEE 1394 camera	$7,000
Computer	PC with 2 RS-232, 4 USB-2 Ports & IEEE 1394 (Firewire)	$3000
Analytical Software	Government developed	$0
TOTAL		**~$69,000**

4 DATA MANAGEMENT AND ZOOPLANKTON DATA COLLECTION PROTOCOLS

Two protocols have been developed by NRL to satisfy the requirements of standardized data collection: the Data Management Protocol and the Zooplankton Data Collection Protocol. They are meant to be used in concert and ensure that system settings are properly set, documented, and recorded. As described below, in many cases, redundant information is recorded in the two major outputs: an Excel Spreadsheet, which contains comments by the operator and lists the settings used, and an *.nd2 file, which contains the diascopic (DIA, transmitted light [brightfield]) and epifluorescent (GFP) image sets. NRL has successfully used both protocols to support its data collections since August 2009.

Implementing the protocols is a three-step process: First, the operator sets up the directories and generates a test-specific Excel spreadsheet. This process is described in Sections 4.1 and 4.3 (step-by-step instructions are in Appendix A). Next, the operator sets up the microscope and collects the image data. This is described in Section 4.2 and 4.3 (and in Appendix A). The operator then runs the analysis routines on the collected image set data, which is described in Section 5.

4.1 Data Management Protocol

The Data Management Protocol serves two purposes. First, it provides a standardized means of storing data with a file naming convention into a directory structure that allows specific-test data to be easily identified. Second, it produces a test-specific spreadsheet that documents key system settings and allows the microscope operator to enter test-specific comments following a review of the image set after it is collected.

4.2 Zooplankton Data Collection Protocol

The purpose of the Zooplankton Data Collection Protocol is to ensure that standardized image data sets are collected. It is used to set the microscope and camera settings prior to data collection and to collect the standardized image sets in an *.nd2 file format following system setup. At this time, only two camera-setting parameters are selected by the microscope operator: the GFP (green fluorescent protein) camera exposure time and the gain settings. Work continues in the refinement of the optimum camera settings for GFP measurements, with the majority of recent data being collected with a camera exposure value of 100 milliseconds (ms) and a gain factor of 1.0. The total magnification is 9.25X (0.5 X objective*10X ocular *1.85X zoom). Many other parameters, such as lamp brightness, are automatically set by configuration files (not editable) accessed by Nikon Elements and are not user-selectable. These meta-data are additionally stored in the *.nd2 file.

4.3 Using the Protocols – Summary Steps

Brief descriptions for using the Data Management Protocol and Zooplankton Data Collection Protocol follow; the complete protocols are provided in Appendix A. The protocols assume that the microscope operator has basic familiarity with the AZ100 microscope and the Nikon Elements Software.

Prior to running the data management and collection protocols, the sample should have been concentrated and stained. A 0.5-mL subsample should have been placed in the sample well and the sample plate placed onto the sample stage. Note that the stained samples should be analyzed within 20 minutes following the 10- minute incubation period.

There is ongoing work to modify the Excel spreadsheet file used in support of this protocol. These modifications are the result of the extensive amount of testing that has been performed in 2010 and 2011. As this is an ongoing process, the Excel spreadsheet that was described in last year's Protocol for Automated Zooplankton Analysis is also described in this document. When the new spreadsheet file is finalized, it will be described in detail in next revision of the Protocol.

4.3.1 Data Management – File naming

The observer starts by creating a new experiment folder for each new *.nd2 file. This folder will contain all images taken for each sample well analyzed during the experiment. A strict file and folder naming convention allows all replicate or subsample data to be associated with the corresponding .xls spreadsheet. The folder should be labeled with the date and a sequential number. For example, a folder Desktop > ANS > 2009 > Month > Day > Treatment > Run Folder could be Desktop > ANS > 2009 > 07_July > 09 > rotifer_300ms > 20090709_rotifer_300ms_001. The file itself should be named to match the run folder.

Once the folder name is established, an Excel data sheet template (.xls or .xlsx) is saved to the folder with the .xls file having the same name as the folder. If necessary, the operator may need to change the Excel security setting to medium to allow Excel to open embedded macros. The operator then opens the Excel worksheet and enters appropriate data for the run. All entries, including redundant data, must be filled before saving. Comments can be added to the worksheet after the .nd2 images are reviewed.

4.3.2 Zooplankton Data Collection

With the camera and both light sources on and the camera connected to the computer, the Elements software is turned on. After the microscope is focused and the optical pathway sent to the camera, the operator opens a live preview in Elements. Clicking the DIA optical configuration on the toolbar allows the operator to focus the camera based on the live preview window. The operator then checks and records the physical microscope settings. The operator then changes to the GFP optical configuration on the toolbar and verifies the GFP settings.

Focusing (by eye or camera) is not trivial due to the depth of the water column, but skilled and experienced microscope operators should be able to accomplish this function. It is important to note that we are recommending a paradigm shift in the way the microscope is operated: the focus is on the complete well, not on individual organisms. Focus becomes more important here, as magnification and the ultimate resolution of the individual organisms is obtained by zooming in on the images rather than on the organisms.

With all settings verified and recorded, the operator selects which folder to save the images in and enters the first filename as "filename_001". The Elements software will automatically increment the number at the end of each run. The operator sets the interval to 1 second and the duration to 31 seconds. (Note that the first image from each microscope modality is deleted to eliminate problems that frequently occur with these two images during data collection). The number of loops is automatically calculated (for 31 seconds, there will be 32 loops because the first loop starts at time zero). A small flag icon should be visible in the loops

tab (see Appendix A, Fig. A-15). This indicates that the routine will be forced to finish when the routine reaches the specified number of loops. If this icon is set on the duration tab, then the routine will be forced to quit at exactly 31 seconds). Finally the operator checks the Lambda tab (which indicates the illumination source) to verify that the first Lambda is DIA and the second is GFP.

The operator then clicks "Run now" to start automatically collecting images of the sample under the ocular. As indicated earlier, this only results in the collection of the image sets. Analysis of the image sets occurs separately after the completion of the image acquisition.

5 DATA ANALYSIS AND BASIC ALGORITHM DESCRIPTION

The Data Analysis algorithms are executed using a zooplankton analysis program as a stand-alone, Windows-based application. (This analysis software will be available at no cost.) When run, the application asks the operator to specify a *.nd2 file to be analyzed. The *.nd2 file associated with a given test is first parsed into brightfield (DIA) and epifluorescent (GFP) image sets. The motility algorithms operate on both of these parsed image sets. The algorithm provides a display of the DIA and GFP image sets as movies and provides the results of image pair analyses (that are conducted to identify motile organisms in both the DIA and GFP image sets) as well as the results obtained across each image set and across the microscope modalities. The routine additionally provides a screen output of the number of living organisms ≥ 50 µm detected in the analyzed data. Additionally, the data analysis routine outputs its final (as well as intermediate) counting results directly into the spreadsheet described in the Data Management Protocol.

Initially, the basic analysis algorithm analyzes the brightfield (DIA) and epifluorescent (GFP) data independently. The algorithm works with image pairs in each microscope modality, in this case, 10 image pairs from both the DIA and GFP image sets. Thresholds are applied to each of these images to create binary images that are subtracted and squared to create new binary images, which depict particles that have moved between the collection of these two images. The number of moving organisms is computed on an image-by-image basis for each of the DIA and GFP sets. Next, these results are weighted by performing analysis across the image sets in each microscope modality and then by analyzing the results across the associated DIA and GFP image modalities (on a spatial bases). The results are then aggregated across the complete image sets to generate the number of living organisms associated with a given analyzed *.nd2 file. As mentioned above, both intermediate and final results are written into the Excel spreadsheet associated with the *.nd2 file analyzed.

Results are written to the Excel spreadsheet associated with the given *.nd2 file that was analyzed. The overall result (the number of viable organisms per mL), which is based on the analysis of the entire image set, is written to the spreadsheet. The software additionally writes out interim results that are generated on an image pair by image pair basis for both the DIA and GFP image sets. Lastly, image data are available for review by the operator. This allows the operator to corroborate automatically generated results and, if required, to amend results based upon their observations of the image set data.

6 FUTURE WORK

Recent work has resulted in an updated protocol, improvements in algorithms, recommendations for sample processing methodology, and recognition that changing thresholds changes our ability to recognize and count motile organisms. There remain ample challenges to accurately counting and determining the viability of organisms in the ≥ 50 µm size class.

Briefly, there is a need to develop an algorithm to count motile organisms ≥ 50 µm and generate error bars for that count. Determining the actual size of organisms ≥ 50 µm is not trivial and should be addressed. Developing an adaptive thresholding algorithm for use with the brightfield microscope modality and refining the one used with the epifluorescent microscope modality will improve detection capabilities in complex samples. As ever, the results of automated methods need to be compared to results of manual counting methods on the same samples. Equally important is conducting the same analyses on complex samples from different locations and perhaps from different applications.

These potential research efforts are discussed more thoroughly in Appendix B, Section 3 and should be considered as the need for repeatable analyses of complex samples with low organism counts increases.

7 REFERENCES

Lemieux, E.J., Nelson, B.N., Riley, S.C., and Robbins, S.H. (2007) Development of Automated Methods for the Enumeration and Classification of Zooplankton and Phytoplankton Samples. Coast Guard Letter Report

Nelson, B.N., Steinberg, M.K., Robbins, S.H., Lemieux, E.J., Herring, P.R., and Drake, L.A. (2010a) Feasibility of Developing a Protocol for Automated Protist Analysis. Coast Guard Letter Report: CG-D-02-11, Accession Number: ADA535257

Nelson, B.N., Steinberg, M.K., Lemieux, E.J., Herring, P.R., and Drake, L.A. (2010b) Analysis Protocol for Automated Zooplankton Analysis. Coast Guard Letter Report, Report: CG-D-01-11, Accession Number: ADA535255

Steinberg, M.K., Lemieux, E.J., and Drake, L.A. (2011a) Determining the viability of marine protists using a combination of vital, fluorescent stains. Marine Biology. 158:1431-1437

Steinberg, M.K., Lemieux, E.J., Riley, S.C., and Drake, L.A. (2011b) Continuing validation of a method for determining viability in protists using two vital, fluorescent stains. Letter report 6130/1113 published by the US Naval Research Laboratory, Washington, DC

United States Federal Register (2009) Standards for Living Organisms in Ships' Ballast Water Discharged in U.S. Waters; Draft Programmatic Environmental Impact Statement, Proposed Rule and Notice, 74 FR 44631-44672 (28 August 2009). National Archives and Records Administration, Washington, DC

United States Environmental Protection Agency (2010) Environmental Technology Verification Program (ETV) Generic Protocol for the Verification of Ballast Water Treatment Technology, Version 5.1. Report number EPA/600/R-10/146. U.S. EPA ETV in cooperation with the U.S. Coast Guard Environmental Standards Division (CG-5224) and the U.S. Naval Research Laboratory. National Sanitation Foundation International, Ann Arbor, MI, 63 pp.+ appendices

(This page intentionally left blank.)

APPENDIX A. PROTOCOLS FOR AUTOMATED ZOOPLANKTON ANALYSIS

The protocols on the following pages provide step-by-step instructions for creating data files, capturing images of treated samples, and analyzing the collected images. The protocols and accompanying screen images were developed using the equipment described in the preceding text. Steps include file naming conventions, creation of an Excel data sheet, data collection, and analysis of digital images of organisms ≥ 50 µm (nominally zooplankton). The government-developed software for analyzing the collected data is in the public domain and is available from NRL. As mentioned in the main body of this document, the specific spreadsheets that are used in support of this protocol are currently being modified to make data collection easier and more efficient. These changes will be documented and included in the next revision of the Protocols for Automated Zooplankton Analysis.

A.1 Zooplankton Data Management Protocol File Naming and Convention

Create a new experiment folder for every new .nd2 file.

Desktop > ANS > 2009 > Month > Day > Treatment > Run Folder

The Experiment Folder should be labeled with the date and then a chronological number. Examples:

Desktop > ANS > 2009 > 07_July > 09 > rotifer_300ms > 20090709_rotifer_300ms_001 (e.g., replicate or subsample 001)

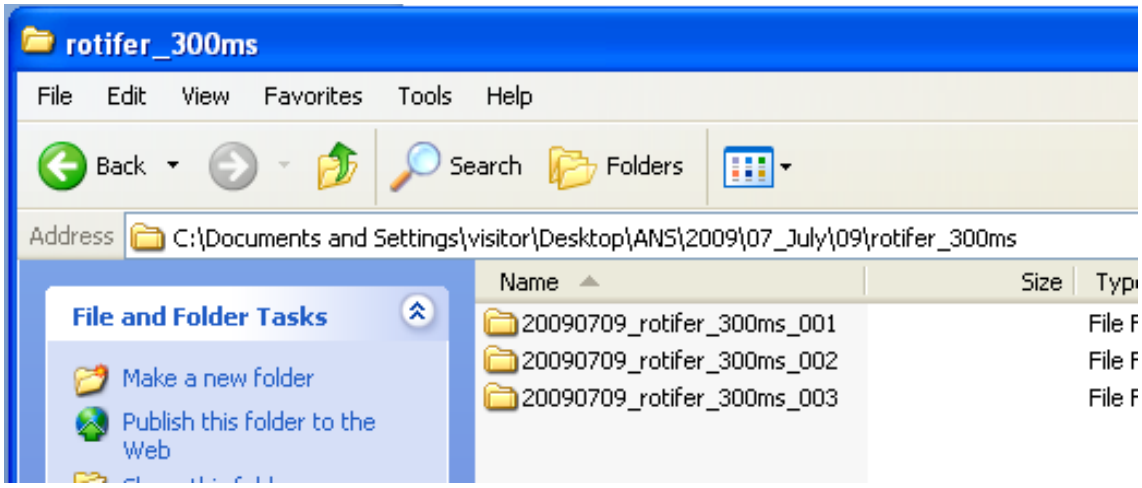

Figure A-1. File naming and convention.

The file itself should be named to match the Run Folder: 20090709_rotifer_300ms_001.nd2 (Figure A-1).

A.2 Zooplankton Data Management Protocol Excel Data Sheet

Each Treatment Folder should have a copy of the AZ100 Excel data sheet.

1. The template will be located in the ANS folder. Save your edited version in the Treatment folder with a filename identical to the sample description (e.g., 20090709_rotifer_300ms.xls; Figure A-2).

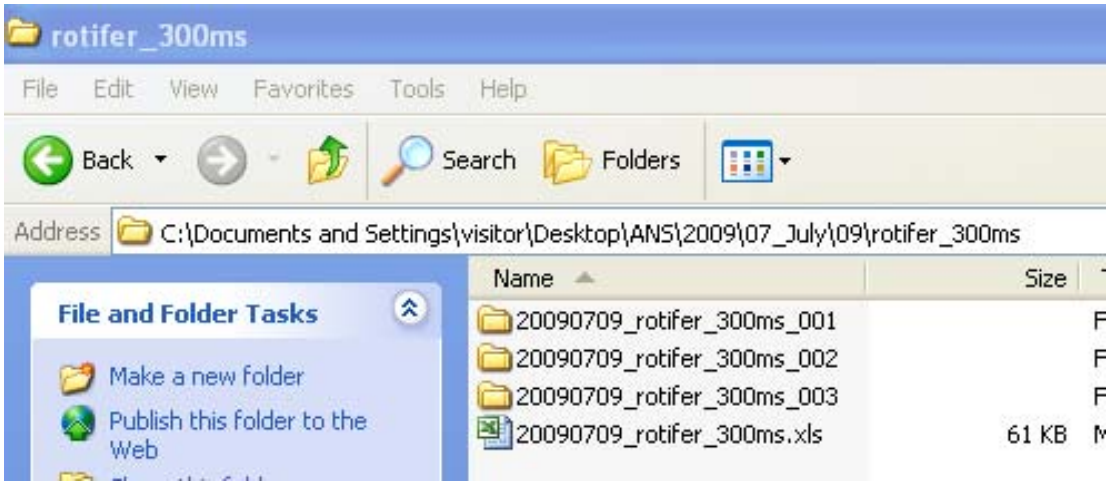

Figure A-2. Excel data sheet in Treatment folder.

2. You may have to configure Excel to be able to open the macro called out in the programs. . If prompted, enable macros to run in the file.

In Excel 2003 running in XP, go to the Tools menu > Macro > Security (Figure A-3, top panel).

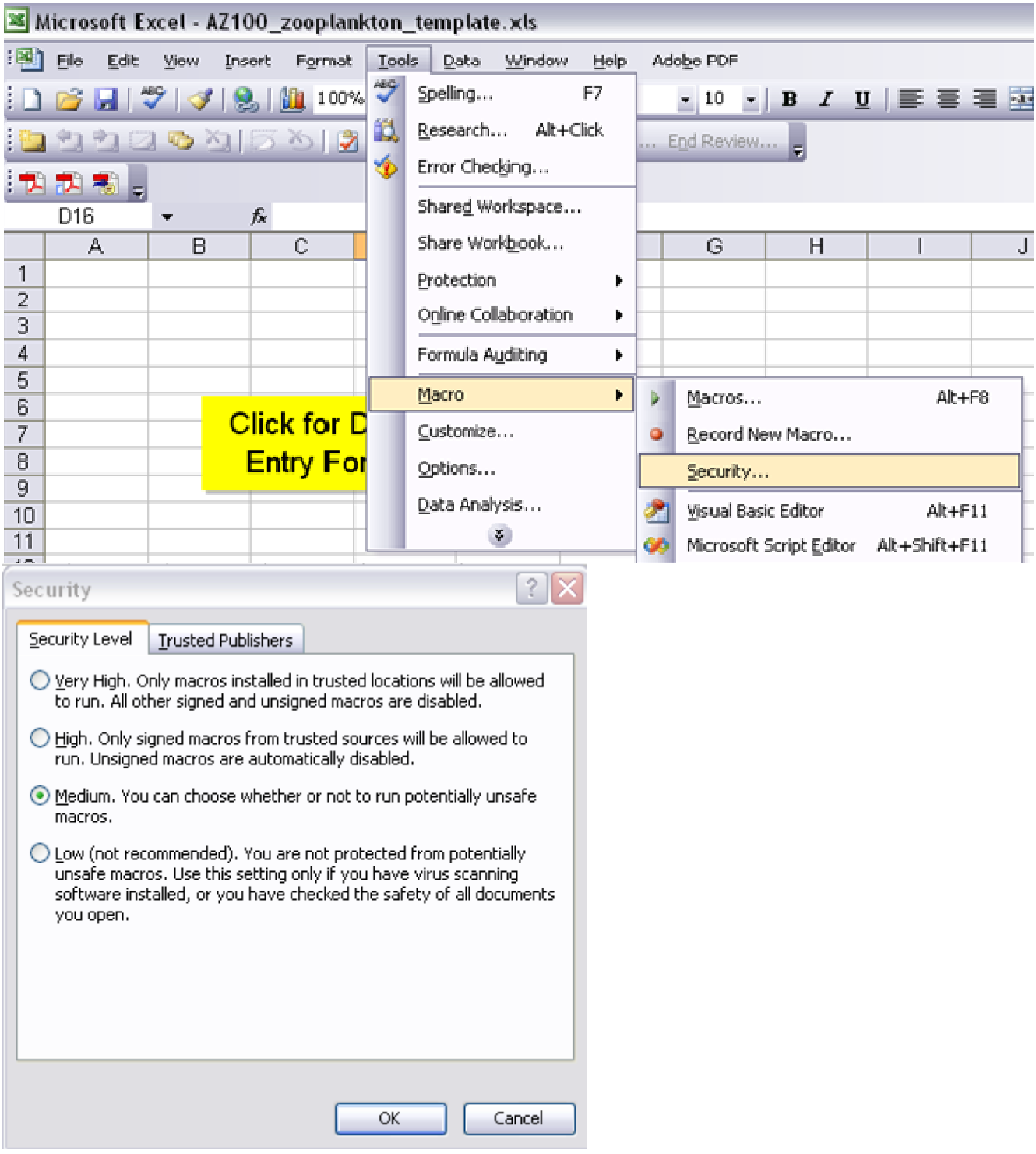

Figure A-3. Excel data sheet security menu (top panel) and security selection level (bottom panel).

Choose Medium security (Figure A-3, bottom panel), click OK, close Excel, and reopen it.

3. On the Data Entry worksheet, click on the yellow Data Entry button (Figure A-4) and complete the form (Figure A-5) with entries or checks as required. Entries or checks are required for all data fields (otherwise, the file cannot be saved.

.

Figure A-4. Data entry form button.

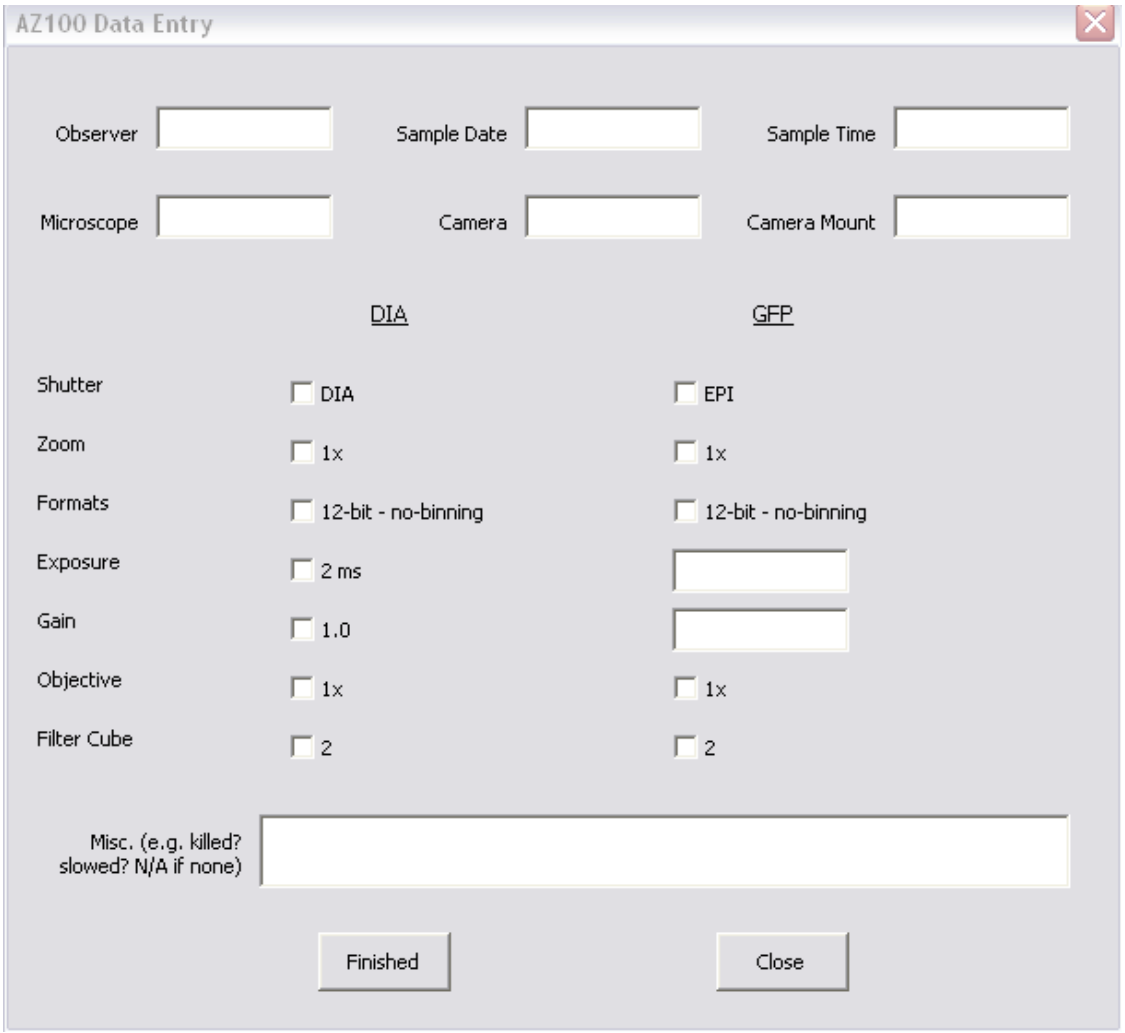

Figure A-5. Required data fields in Excel data sheet. Note that the data entry form will be updated in the final version.

4. Folowing data collection, after each .nd2 file is recorded, view the images and write observations in the appropriate 'Comments' worksheet in the Excel file (Figure A-6).

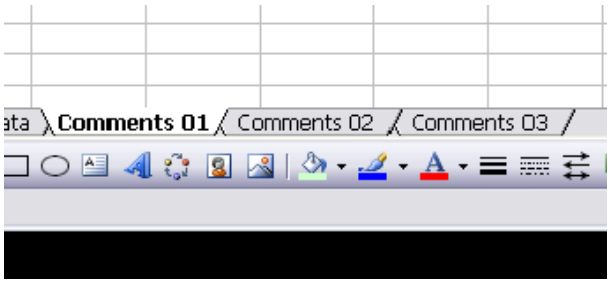

Figure A-6. Excel data sheet comment pages for each .nd2 file. Note that the operator comment section will be modified and updated in the final protocol.

A.3 Zooplankton Data Collection Protocol

A.3.1 Initial Set-up

1) Before opening Elements program, turn on the camera and both lamps.
2) Place your sample on the stage and focus the microscope using the oculars. Once the sample is in focus, pull out the knob to change the optical pathway from the oculars to the camera.
3) In Elements, open up a live preview. You can do this by going to the Acquire menu (Live – Fast), then by clicking the "Play" Icon on the toolbar (Figure A-7), or by pressing the '+' key on the keyboard.

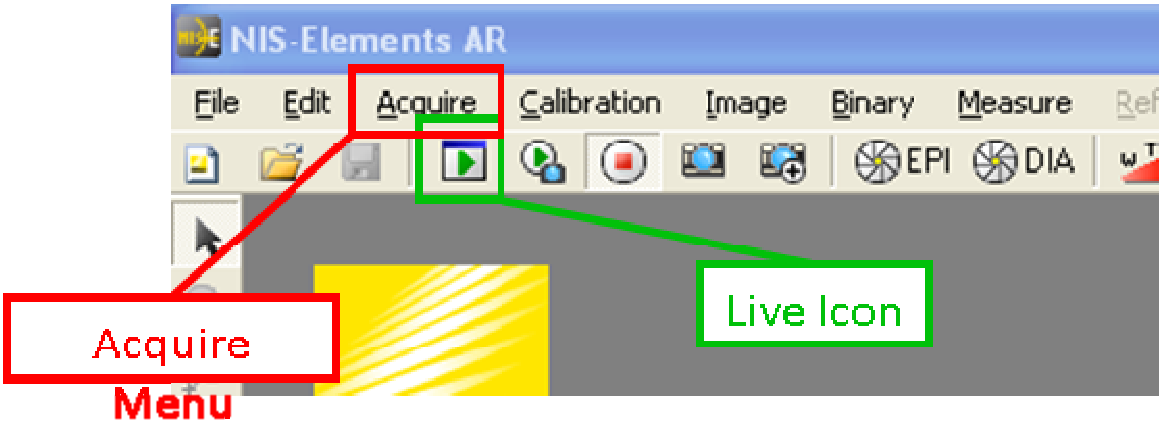

Figure A-7. Acquire menu and Live icon.

A.3.2 Camera Set-up

4) Click on the "DIA" optical configuration in the toolbar, *not* the shutter control button (Figure A-8).

   The optical configuration buttons are preset with settings for the camera exposure, image gain, and objective lens and filter cube, etc.

   These settings will be used when you take a time series of images.

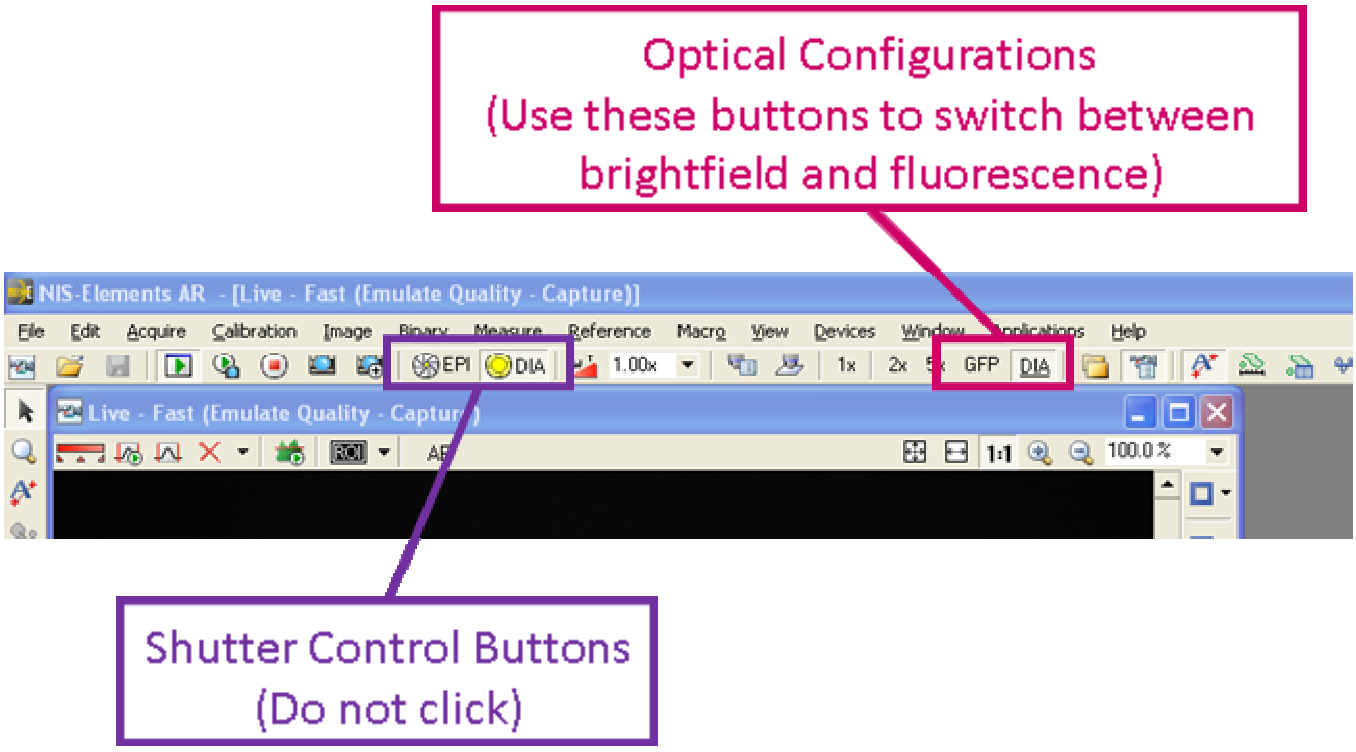

Figure A-8. Optical configuration and shutter control buttons.

A.3.3 Camera Focus and Settings

5) Now use the focus ring on the coupler to focus the camera based on the Live Preview window. You may need to zoom in on the Preview to make sure it is as crisp as possible (Figure A-9).

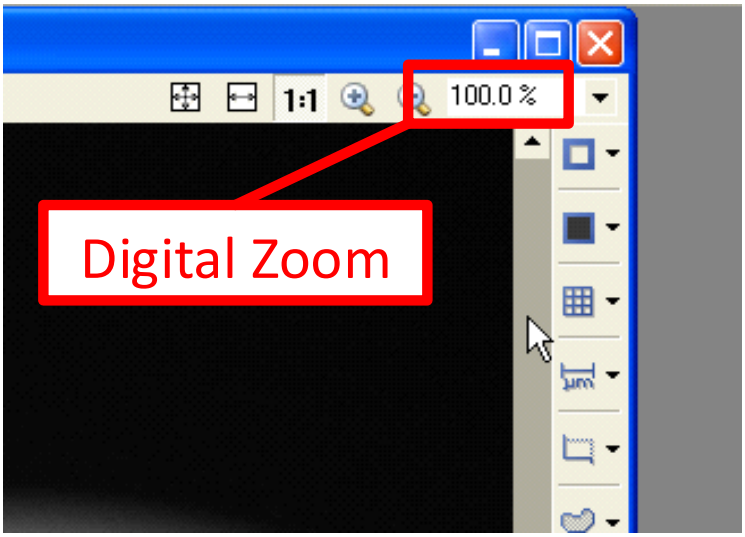

Figure A-9. Digital zoom button.

6) Check the physical microscope settings:

- The GFP Band Pass filter cube is in place (2)
- The 0.5X objective is in place.
- Zoom is at 1.85X.
- At the bottom right of the base, the ND16 and ND8 filter knobs are pulled out (keep the ND2 knob pushed in; n.b. ND = neutral density).

A.3.4 Verification of Elements Settings - DIA

7) Check all your Elements settings. With the DIA optical configuration button (orange box, Figure A-10) selected, there are several things to look for (Figure A-11):

i. Microscope camera status is set to live.

ii. The zoom is 1.85X.

iii. The formats for live and for capture are both set at 12-bit - no binning.

iv. The exposure time is 5 ms.

v. The hardware gain is 1.0.

vi. On the manual microscope pad menu, the 0.5X nosepiece is selected.

vii. The DIA filter turret (the first gray icon) is selected.

(Note that some of these settings may change over time and depending on the sample.)

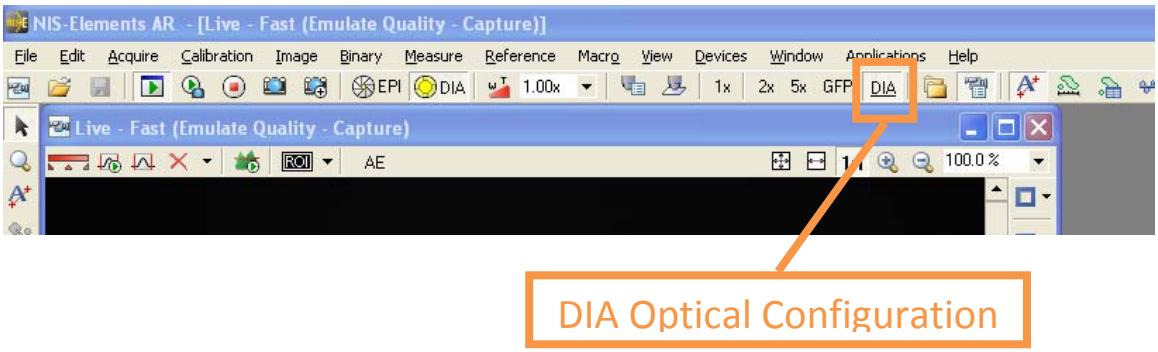

Figure A-10. DIA optical configuration button.

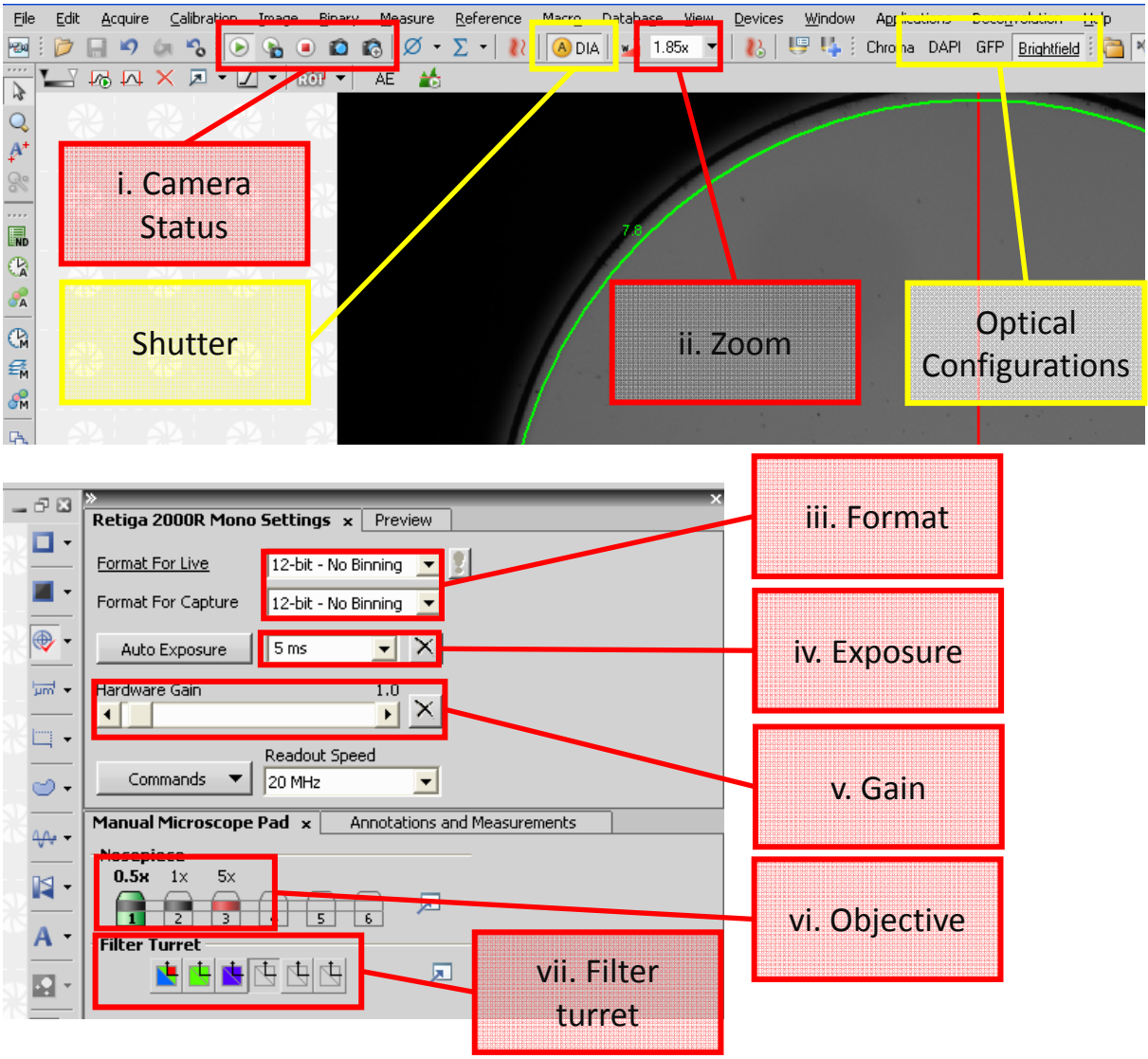

Figure A-11. DIA setting summary.

A.3.5 Verification of Elements Settings - GFP

8) Now click on the GFP Optical Configuration button (green box, Figure A-12) and double check the GFP settings:

- The EPI shutter button is selected.
- The zoom is 1.85X.
- The formats for live and for capture are both set at 12-bit - no binning.
- The exposure time is 100 ms.
- The hardware gain is 1.0.
- On the manual microscope menu, the 0.5X nosepiece is selected.
- The GFP filter turret is selected.

(Note that some of these settings may change over time and depending on the sample.)

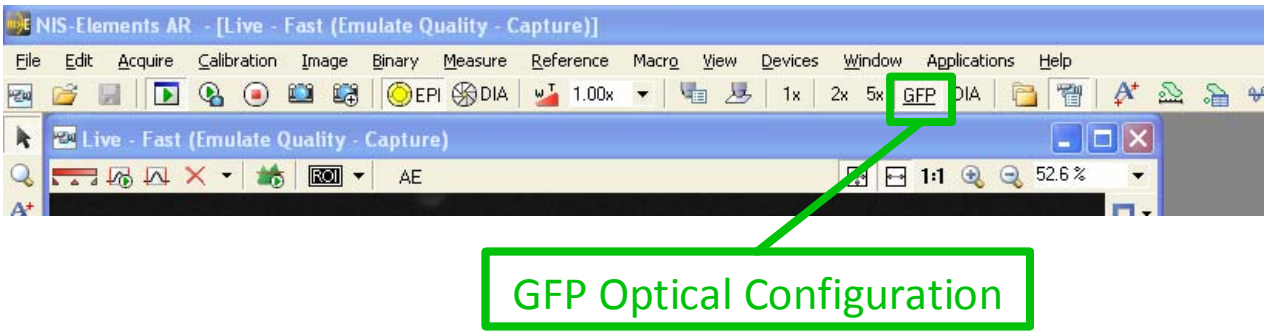

Figure A-12. GFP optical configuration.

If you make changes to the exposure or gain while an Optical Configuration is selected, the new values will be propagated as part of the preset values. That is why you *must* ensure the settings are correct before beginning data collection.

A.3.6 Manual Changes

You can reset the presets by manually changing the above settings, going to the Calibration menu at the top of the screen, and choosing Optical Configurations. Make sure the correct optical configuration is selected in the menu to the left and then click on "Assign Current Camera Settings" or "Assign Current Microscope Settings" (Figure A-13).

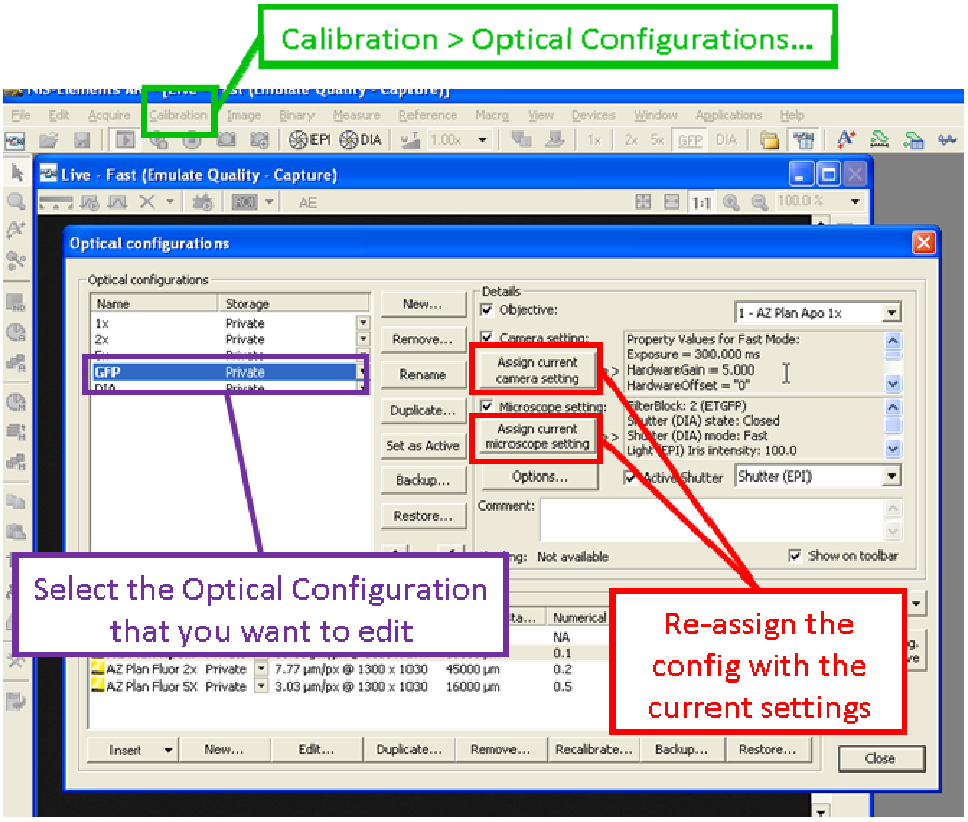

Figure A-13. Assign current settings.

A.3.7 Final Step - Designating Acquisition File

9) At this point, you have checked all your settings and the camera is in focus. Go to the Applications menu and choose Define/Run Experiment (Figure A-14).

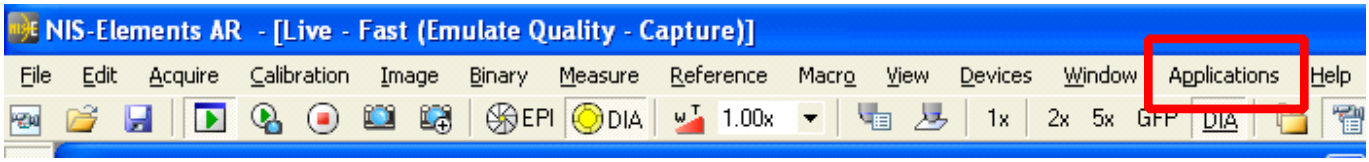

Figure A-14. Applications button.

10) Click on Browse to choose the directory to save files. Enter your file name followed by 001 (Elements will automatically increase the number at the end of each run). Set the Interval to 1 sec and the Duration to 31 sec. The Loops will automatically change to 32. Assure that the flag icon appears in the Loops tab. If the icon is in the Duration tab, click on the Loops tab to move the flag icon (Figure A-15).

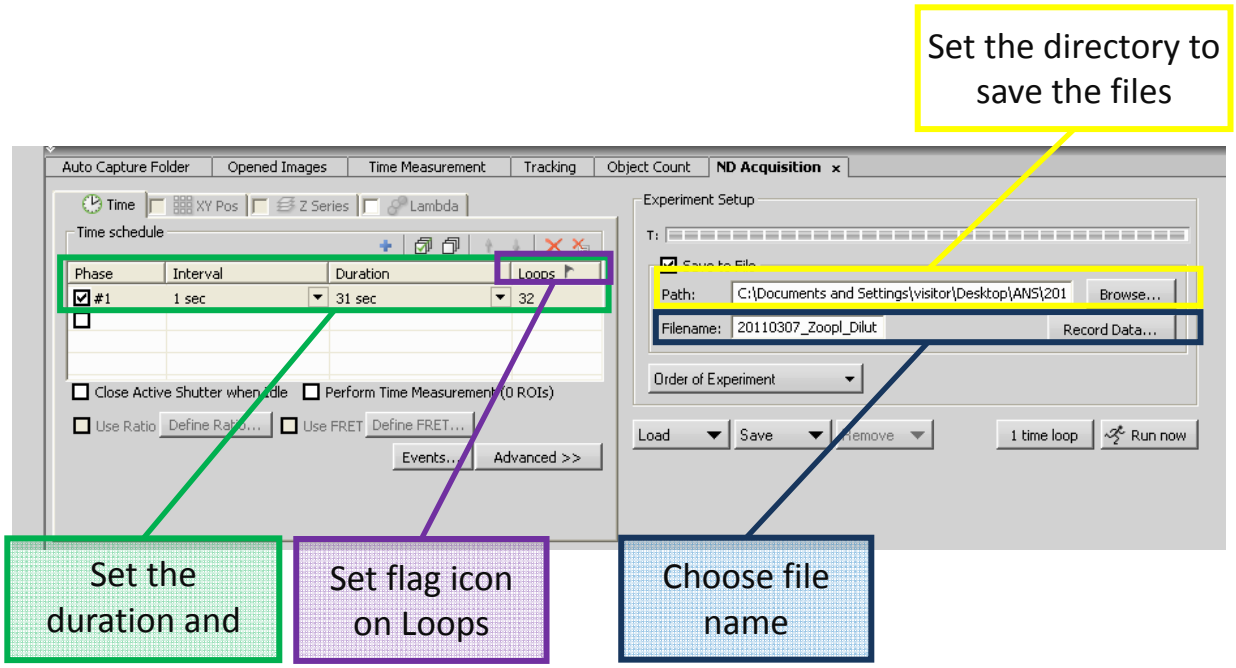

Figure A-15. File designation set-up.

A.3.8 Acquire Data

11) Click on the Lambda tab (Figure A-16). Make sure the first Lambda is DIA and the second Lambda is GFP.

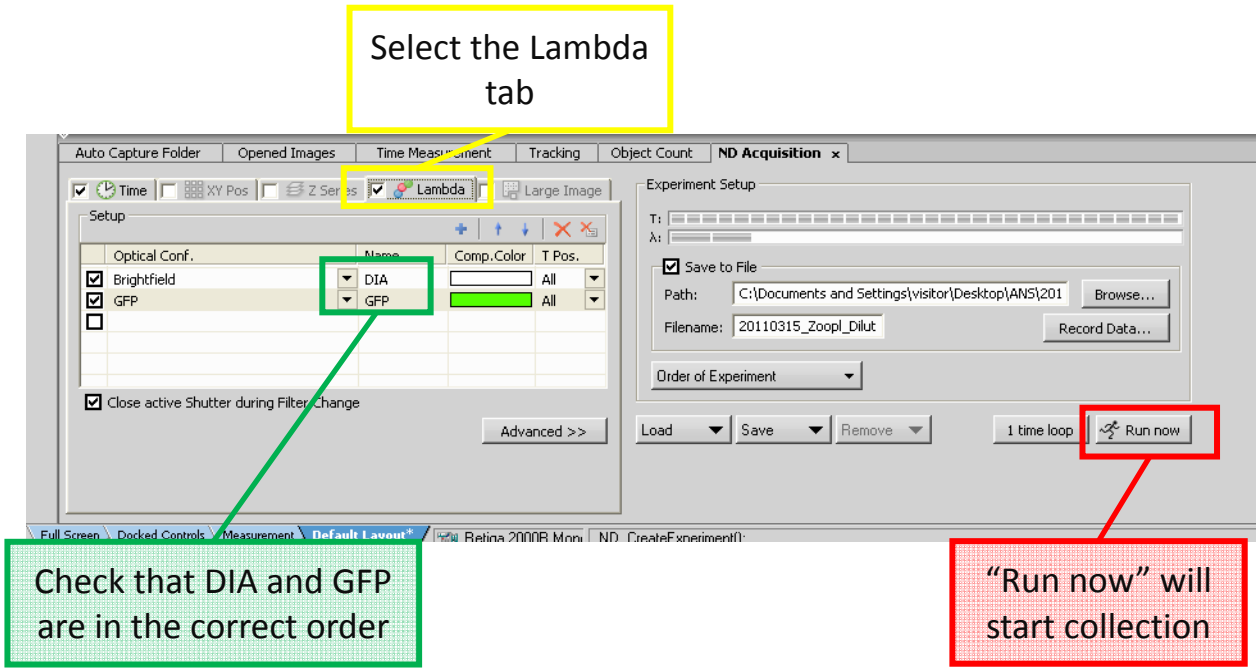

Figure A-16. Setting light source sequence.

12) Everything is set up, so click "Run now" to start the time series (Figure A-16).

When all samples have been processed and the data acquired, data processing proceeds as described in Section 5 of the main report.

APPENDIX B. RESULTS FROM ONGOING RESEARCH AND DEVELOPMENT

Ongoing research and development activity in support of this and parallel efforts has affected this protocol and its finalization. The major emphasis of automation research and development conducted in last fiscal year was the generation and evaluation of a significant number of complex samples that are more representative of those that would be encountered during the evaluation of BWMSs. The large number of samples evaluated this year afforded a unique opportunity to observe and assess the performance of the automation algorithms under the variety of conditions that were either simulated or tested during these ongoing activities. It also provided a unique opportunity to observe a significant amount of time-resolved microscope image data of complex samples taken using this protocol.

The most significant impact of ongoing research and development activities is related to the recommended method for holding samples under the microscope. The results of this work, detailed in this Appendix, are that unruled Sedgwick Rafter counting chambers are recommended as the primary method for holding samples in the ≥ 50 µm size class under the microscope.

The complexity of samples makes the accurate enumeration of organisms difficult. The algorithms initially work independently on both the brightfield and epifluorescent imagery by analyzing 10 successive image pairs (from each microscope modality) collected twenty seconds apart. That is, the first image pair consists of the image collected at time 1 s (second) and the image collected at 21s, and the second image pair consists of the image collected at time 2 s and the image collected 22 s, etc. In the case of motile organisms, this approach should result in 20 discrete signals for each organism in each microscope modality's output "motility" image. The result is a binary image showing the motion paths of motile organisms. In both microscope modalities, many organisms are only observed occasionally as they move through the sample. This can result from debris obscuring organisms (brightfield) and from weak signatures (both brightfield and epifluorescent) associated with certain organisms. In the case of weak signatures, it has been observed that decreasing the thresholds used in motility algorithms can result in an increased number of observed organisms. These issues make the accurate enumeration of organisms using automated methods an area of ongoing research and development.

Another important observation is related to determining the size of observed motile organisms in complex samples. In samples that have a significant number of motile organisms, it may not be possible to precisely determine the size of all of the observed organisms. First, the apparent size of organisms in the epifluorescent microscope modality is impacted by the thresholds used to observe the data. As many organisms are uniquely detected using this microscope modality, determining the size of these organisms may not be possible without brightfield corroboration. Second, during automated analyses, the microscope focus is fixed. Consequently, the apparent size of organisms can change as they move vertically through the water column. Third, during manual microscopic analyses, size can only be accurately determined by focusing on every observed organism. This may not be feasible in reasonable observation times in a complex sample with many motile organisms.

The final section of this Appendix will provide the status of the Automated Zooplankton Analysis efforts and provide recommendations for performing additional research and development directed at finalizing both the automation algorithms and overall protocol.

B.1 Unruled Sedgwick Rafter Counting Chambers Versus Sample Well Trays

In the 2010 Protocol for Automated Zooplankton Analysis, SensoPlate™ Glass Bottom Cell Culture Plates were recommended for holding samples in the ≥ 50 µm size range under the microscope. This sample well tray contains 24 wells, approximately 16 mm in diameter that are suitable for containing 0.5 mL to 1.5 mL of fluid. NRL limited the sample volume to 0.5 mL to reduce depth of focus issues in the microscope. This type of sample well tray was thought to be ideal since, with the microscope's automated X-Y stage, each of the 24 wells could be prepared simultaneously and the majority of these wells analyzed sequentially.

Figure B-1 provides two images. The image on the left is a brightfield microscope image showing the entire sample well. The image on the right shows the epifluorescent microscope image generated from this same sample well. A review of the images shown in Figure B-1 demonstrates the primary reason why the SensoPlate™ Glass Bottom Cell Culture Plates were recommended for use with samples in the ≥ 50 µm size class. The brightfield image of the sample well shows a defined sample well boundary and no optical distortions or occluded areas. The epifluorescent image also shows no distortions or occlusions that would prevent the reliable detection of fluorescent objects within the sample well.

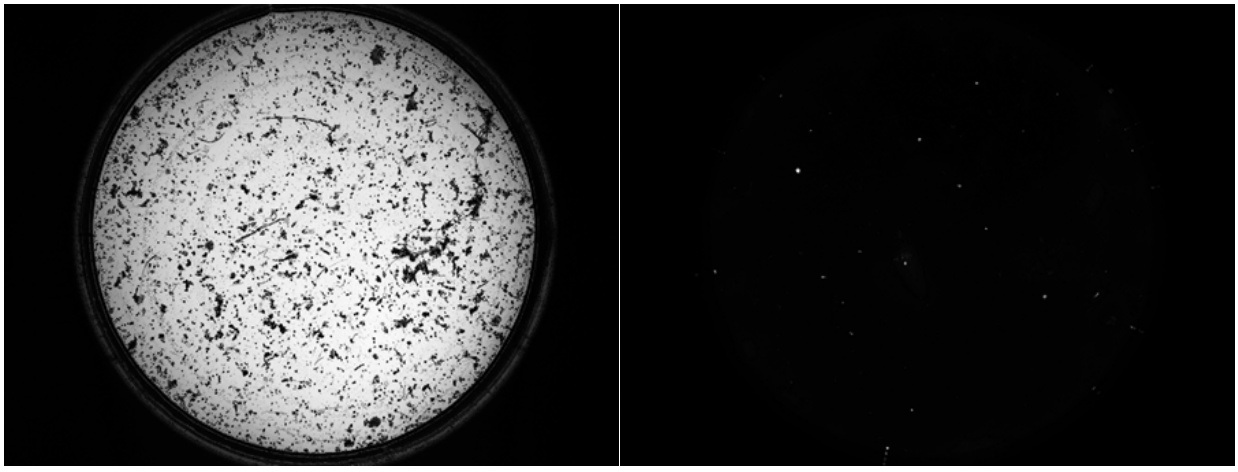

Figure B-1. Brightfield (left) and epifluorescent (right) images collected of a complex sample using a sample well tray. These images show little or no distortions of either the brightfield or epifluorescent image data.

In comparison, Figure B-2 provides two images that were collected from a different sample well. The image on the left is a brightfield image showing the entire sample well. The image on the right shows the epifluorescent microscope image generated from this same sample well. A review of these images shows that the outer perimeter of brightfield image of the sample well is distorted. These distortions are significant and result in the imaged area in the well not appearing circular. Further, the outer perimeter of the well from approximately the 6 o'clock to 3 o'clock positions is occluded because of these distortions. This would preclude the ability to observe organisms in this sample well region in the brightfield image data. The epifluorescent image data, however, does not show the same distortions and occluded areas as the brightfield image data. It was thought that the majority of organisms that could not be observed in the occluded regions of the brightfield imagery could still be observed in the epifluorescent image data. As a result of this, work continued using the SensoPlate™ Glass Bottom Cell Culture Plates to hold samples under the microscope.

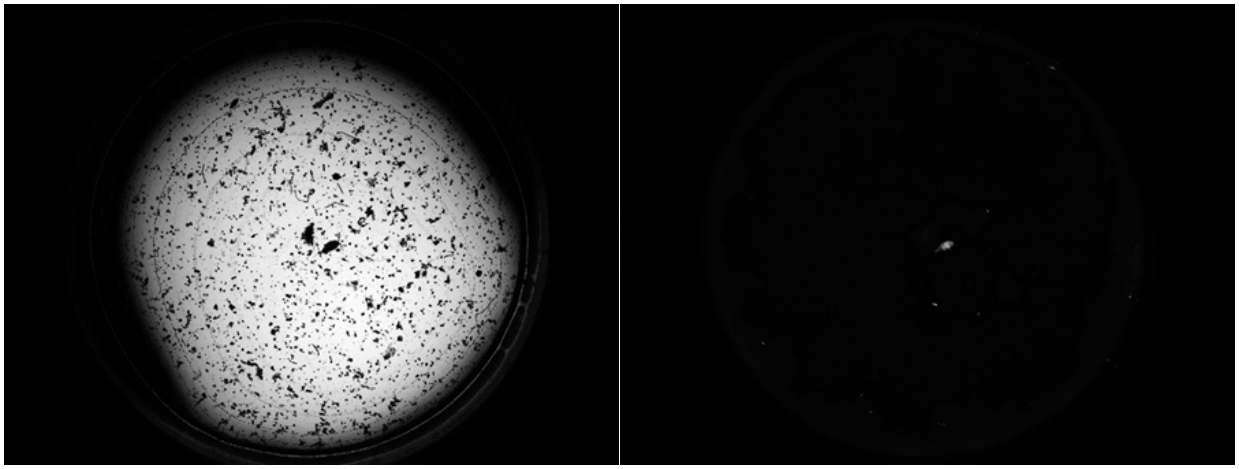

Figure B-2. Brightfield (left) and epifluorescent (right) images collected of a complex sample using a sample well tray. The brightfield image shows distortions that occlude areas in the well. The epifluorescent data does not show a similar distortion.

Initially it was thought that the observed distortions and occlusions resulted from menisci that formed because of surface tension between the well's walls and the fluid sample. Additional work has since shown that the observed distortions and occlusions are more likely the result of a misalignment in the microscope optics. As the microscope used is fairly sophisticated and regularly serviced by manufacturer's representatives, it is felt that the type of alignment issues that result in distortions and occlusions of regions of the sample well may be endemic when using similar microscope equipment. However, it was also thought the automation algorithms could compensate for these types distortions and occluded areas in the brightfield imagery.

Efforts were continuing in support of the Protocol for Automated Protist Analysis, and it was decided to build upon that work. . In support of the protist work, 20 µL sample volumes were being evaluated using unruled Sedgwick Rafter counting chambers. To make this approach more applicable for holding samples in the ≥ 50 µm size range, it was decided to adjust the objective lenses and added zoom level to image a 0.5 mL sample volume or half of the area of an unruled Sedgwick Rafter counting chamber.

Figure B-3 provides two images generated from an unruled Sedgwick Rafter counting chamber. The image on the left is the brightfield image. The image on the right is the epifluorescent image. A review of these images shows that there are no distortions causing occlusions in the brightfield image (note that the lack of a straightedge in the upper left portion of the image is a distortion believed to be caused by microscope alignment, but it does not cause the sample region to be occluded). The epifluorescent data is similarly without distortion or occlusions. The images shown in Figure B-3 are typical of the over 100 samples that have been observed in unruled Sedgwick Rafter counting chambers.

The major disadvantage of using unruled Sedgwick Rafter counting chambers is that the whole chamber cannot be imaged simultaneously while providing the less than 10 µm resolution that is required when working with samples in the ≥ 50 µm size class. Obtaining this spatial resolution can only be achieved when imaging half of the unruled Sedgwick Rafter counting chamber, that is, a 0.5 mL sample volume. Consequently, motile organisms can swim out of the field of view during the 31-second observation window.

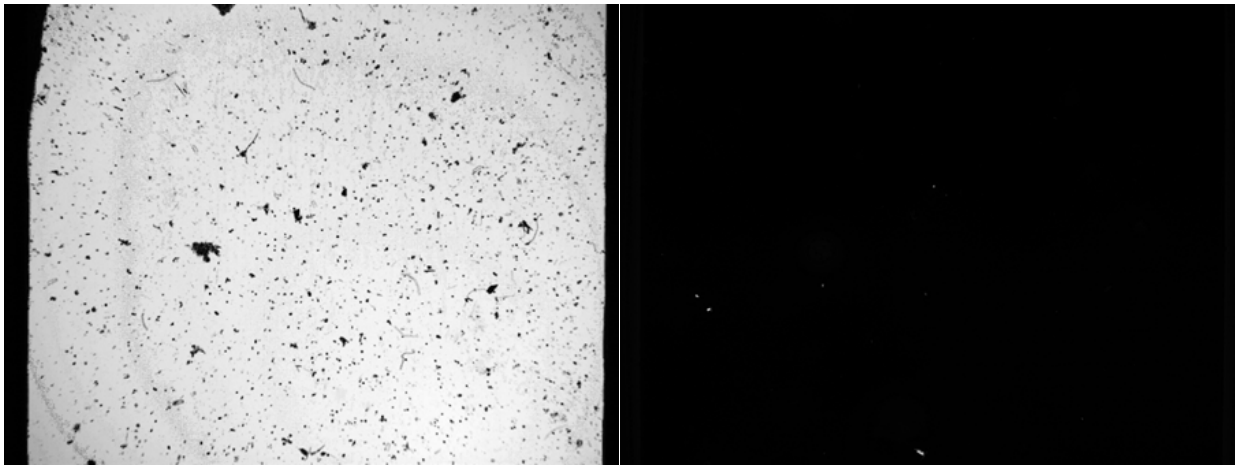

Figure B-3. Brightfield (left) and epifluorescent (right) images collected of a complex sample using an unruled Sedgwick Rafter counting chamber. The microscope's magnification was adjusted to image half of the chamber (0.5 mL). No distortions or occlusions have been observed in samples in Sedgwick Rafter counting chambers.

A second issue with sample well trays was additionally observed (Figure B-4). Both these images were generated from epifluorescent image sets. Shown in both cases is the automation algorithm's output "motility" image generated from the epifluorescent image data. This image is generated by the analysis of 10 image pairs that are collected 20 seconds apart. The image on the left was generated from a sample contained in a SensoPlate™ Glass Bottom Cell Culture Plate. The image on the right was generated from a sample in an un-ruled Sedgwick Rafter counting chamber.

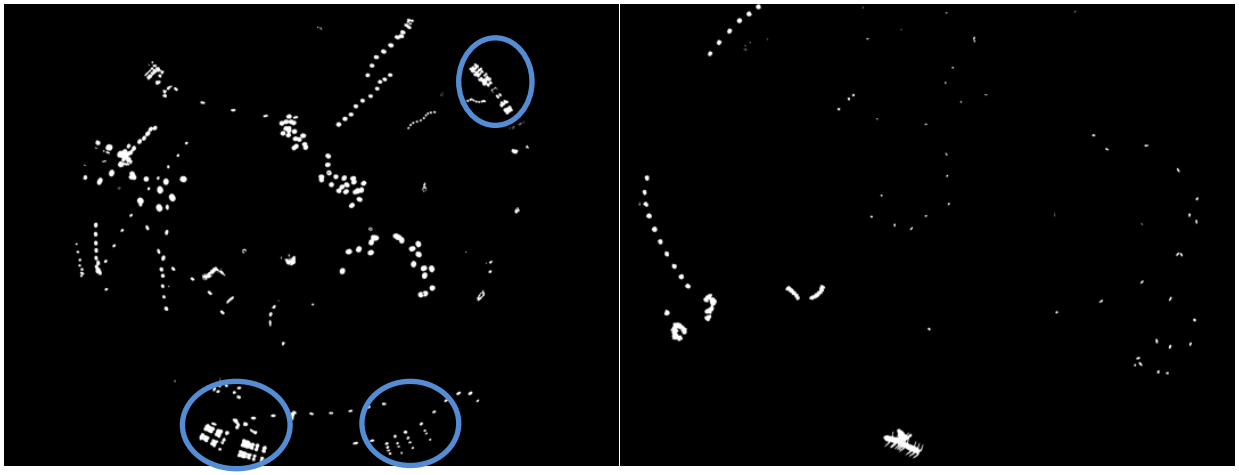

Figure B-4. Epifluorescent "Motion" Images. The motion image on the left was collected from a sample well on a sample well tray (with chamber wall reflections circled in blue ellipses). The image on the right was collected from an unruled Sedgwick Rafter counting chamber. Note there are no wall boundary effects (such as residual signals or multiple organism reflections) observed in the unruled Sedgwick Rafter counting chambers. Also note that organisms that swim into or out of the field of view are "tracked" by the automation algorithms.

The three blue ellipses shown on the image on the left in Figure B-4 show where reflections from the well boundaries have created multiple signals from single organisms as they move through the microscope's field of view. (Note that there are other reflections not highlighted in this Figure.) This type of reflection at the sample holder's external boundary has not been observed in samples that are held using unruled Sedgwick Rafter counting chambers as shown on the right,. It was thought that the effect of these reflections could be eliminated or reduced by applying an appropriate mask to "block" out the outer region of the sample well tray. However, this approach requires the sample to be accurately placed on the microscope in order for the mask to either properly eliminate these reflections or for it not to block off a region of interest within the sample well.

The major advantage of the SensoPlate™ Glass Bottom Cell Culture Plates is that the entire sample well can be imaged simultaneously with required spatial resolution. The disadvantages of the SensoPlate™ Glass Bottom Cell Culture Plates are distortions that create occluded regions in the sample wells in the brightfield imagery and reflections from fluorescent organisms at the well boundaries creating spurious fluorescent signals.

The major advantage of the unruled Sedgwick Rafter counting chambers is that they provide a means to collect brightfield and epifluorescent image sets that are not impacted by distortions, occlusions, and spurious reflections. The unruled Sedgwick Rafter counting chambers produce superior quality image sets in both microscope modalities. The major disadvantage of using unruled Sedgwick Rafter counting chambers is that organisms can move out of the field of view during the observation window. Because these organisms can be "tracked" as they move into and out of the field of view (and organisms can be missed due to other causes including sample complexity regardless of how they are held under the microscope), it is felt that the advantages of improved image quality far outweigh the disadvantage of organisms moving out of the field of view during the observation window. It is also felt that the costs for developing a custom reduced volume Sedgwick Rafter counting chamber cannot be justified at this time.

Therefore, as a result of the ongoing research and development activities conducted under this year's program, the use of standard sized unruled Sedgwick Rafter counting chambers is now recommended for holding samples in the ≥ 50 µm size class under the microscope. For this size class, it is recommended that half of the chamber (a 0.5 mL sample) be imaged. The microscope's X-Y stage can be adjusted following the collection of an image set to allow the ability to image two 0.5 mL samples per Sedgwick Rafter counting chamber thus imaging the complete chamber . Further, a custom slide holder can be used to position multiple, un-ruled Sedgwick Rafter counting chambers under the microscope simultaneously. The X-Y stage can then be utilized to collect image sets sequentially of appropriate areas on each of the unruled Sedgwick Rafter counting chambers.

B.2 Sample Complexity – Organism Enumeration and Size Determination

Samples associated with the ≥ 50 µm size class during tests of BWMSs are inherently complex. This is the result of particulate, dissolved organic, and mineral matter that are added to the challenge water. This problem is made worse by the need to concentrate samples expected to contain less than 10 viable organisms ≥ 50 µm in size per m^3 prior to making measurements. To reliably detect organisms ≥ 50 µm at the expected density of less than 10 per m^3 requires that samples be concentrated up to 60,000:1 to ensure their detection. However, at this concentration factor, samples can become opaque. Work performed by NRL has indicated that samples at challenge water levels of particulate loading can be concentrated up to 600:1 and still have sufficient transmission for observing organisms in the sample. The tradeoff is that the

reduction in concentration level in turn creates requirements for observing more sample volume to ensure samples have less than 10 viable organisms per m^3.

The majority of ballast water samples evaluated during BWMS testing can also have a significant concentration of viable organisms. This is true for both initial test and control fill operations (where challenge levels of organisms must be present) but also for evaluating samples from the control tank that have not been exposed to the treatment technology.

This year's ongoing research and development effort explored the effect of sample complexity on the ability to both accurately enumerate and determine the size of detected motile organisms in the ≥ 50 µm size class.

The algorithms that are used to detect motile organisms in complex samples are fairly detailed. The algorithms initially work independently on both the epifluorescent and brightfield imagery by analyzing 10 successive image pairs from each microscope modality that are collected twenty seconds apart. For each image pair, binary images are generated from each of the two images that make up the image pair and subtracted and squared. This subtraction ideally results in two objects for every organism that moved (and no objects for organisms or objects that did not move) in the twenty-second interval between when the two images making up the pair were collected. Summing the results across the ten image pairs creates a "motility" output image for a given microscope modality that tracks the positions of the motile organisms across the 10 image pairs analyzed. Ideally, 20 discrete signals should be generated for each motile organism in each microscope modality's output "motility" image. Additionally, the results across the two microscope modalities can be combined to create a single "motility" output image. This image should ideally have 40 discrete signals for each motile organism.

The automation algorithm that operates on the epifluorescent image data uses both image normalization and adaptive thresholding methods to create the binary images that are subtracted. This approach affords the ability to identify motile organisms with weak epifluorescent signals. The algorithm that operates on the brightfield microscope modality uses image normalization but does not currently utilize adaptive thresholding methods to create binary images. In both microscope modalities, many organisms are only observed occasionally in the 10 image pairs that are analyzed. This can result from debris obscuring organisms (brightfield) and from low contrast or weak organism signatures (both brightfield and epifluorescent). In the case of weak epifluorescent signatures, it has been observed that in many cases decreasing the thresholds used in motility algorithms can result in an increased number of observed organisms. In addition, if an organism is a "slow mover" it may not move sufficiently to create two discrete signals. All of these types of issues make it more difficult to accurately enumerate the number of organisms based on analyses of the algorithm's output "motility" images.

Accurately determining the size of detected motile organisms can also be difficult. First, there is quite often a significant difference in the size of observed organisms in the brightfield versus epifluorescent microscope modalities. Further, the size of objects in the epifluorescent microscope modality is impacted by the thresholds used either to create a binary image or to display the image data. Some organisms are observed in only one of the two microscope modalities. Many organisms are uniquely detected in the epifluorescent microscope modality and as such, it may be impossible to accurately determine their size without brightfield microscope corroboration.

Even with the larger size class organisms (≥ 50 µm), depth of focus limitations and the 1 mm water column (the depth of the unruled Sedgwick Rafter counting chambers) create some issues. When using automated analysis methods, the microscope focus is fixed. Consequently, the apparent size of organisms can change

as they move through the water column in both microscope modalities. When using manual microscopic methods, size can only be accurately determined by focusing on every observed organism. This may not be feasible within reasonable observation times in a complex sample with many motile organisms.

Two examples of motility analyses performed on samples with organisms ≥ 50 µm are provided to illustrate issues associated with accurately enumerating and determining the size of organisms in complex samples.

Figure B-5 provides images of a relatively simple sample with organisms ≥ 50 µm that were generated during a simulated, small-scale treatment experiment performed in April 2011. The image on the left is the algorithm's output motility image generated from the brightfield image data. The image on the right is the algorithm's output motility image generated from the epifluorescent image data. A review of these images shows only one organism that was uniquely detected by only one microscope modality. The motion path associated with this organism is circled in blue in the epifluorescent motility image. Two organisms in this sample were "slow movers". These organisms are circled in red in the figures. These organisms produce a motion path that is more "line" like than typical discrete signatures that are obtained from more motile organisms. Even in the case of "typical" motile organisms in these data, there is rarely a case where two objects were produced for each of the image pairs analyzed.

A review of the images in Figure B-5 also shows that the size of the signals produced by the motile organisms is very different for the brightfield versus epifluorescent microscope modalities. This discrepancy demonstrates the problem of accurately determining size with this type of microscope data.

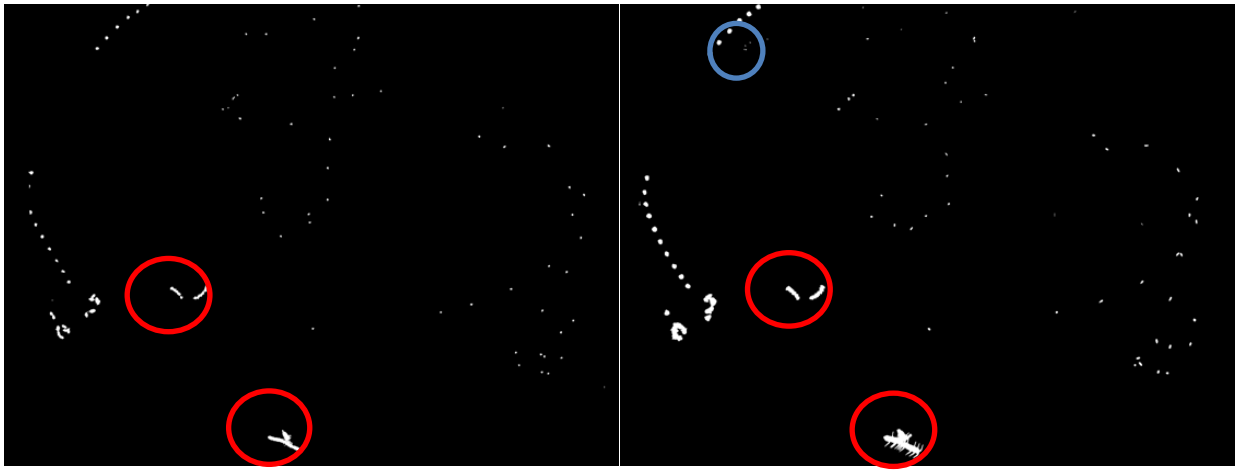

Figure B-5. Automated algorithm "motility" output images for the brightfield microscope modality (left) and for the epifluorescent microscope modality (on right). One organism was uniquely detected in the epifluorescent microscope modality (circled in blue). Organisms that moved slowly and which consequently produced "lines" instead of "dots" are circled in red.

Figure B-6 shows the algorithm's composite output motility image (i.e., combined images). In this image, the paths of the organisms are better defined than in the individual microscope modality output motility images. The issues with the observed organism's size being different in the two microscope modalities are easily observed in the organism path that is circled in blue. As was the case with the individual microscope modality output motility images, very few if any of these organisms produced the 40 independent signals that could be produced if motile organisms produced the expected two signatures for each image pair analyzed.

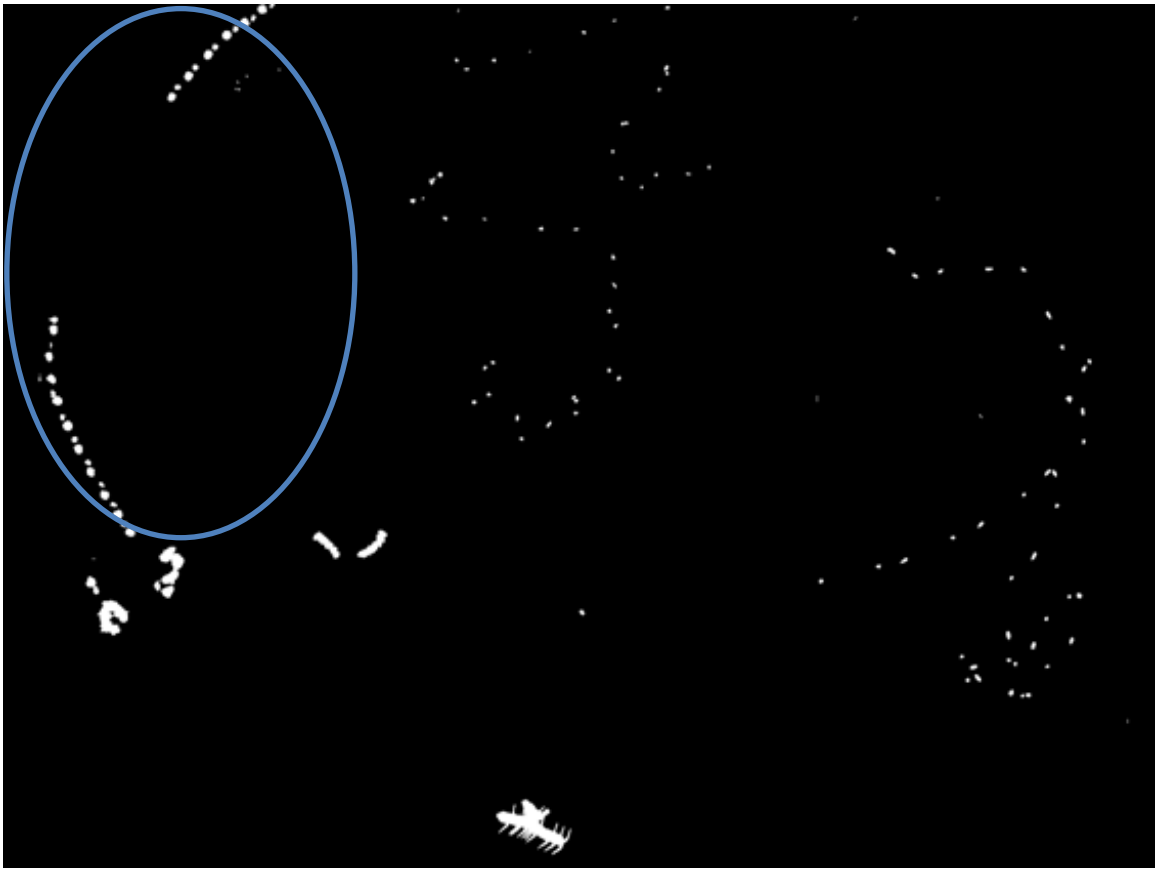

Figure B-6. Automated algorithm composite "motility" output image. Note most of the organisms detected in this image did not create 40 independent signals. This makes it more difficult to enumerate the number of viable organisms even in this sample that had a relatively small number of motile organisms. The difference in size between objects detected in both microscope modalities is easily observed in the organism path that is circled in blue.

A review of the data provided in Figures B-5 and B-6 show that even with this relatively simple sample, accurately determining the viable organism count is not trivial based on an analysis of these output images. There is more than one possible interpretation for the number of organisms that created the motion paths shown in these images.

Figure B-7 provides an example of a more complex sample with organisms ≥ 50 µm that were generated during the same simulated small scale treatment experiment performed in April 2011. The image on the left is the algorithm's output motility image generated from the brightfield image data. The image on the right is the algorithm's output motility image generated from the epifluorescent image data. This sample was more complex because it had both a higher number of motile organisms and a higher amount of debris loading (compared with the sample that was shown in Figures B-5 and B-6). A review of the two images provided in Figure B-7 shows the majority of the detected organisms were detected in both microscope modalities, with only one or two organisms detected uniquely by each of the microscope modalities. The motion paths of these organisms are much more complex and there is a significant variation in the size of the observed organisms in each of the individual microscope modalities. Many organisms were additionally observed only once or twice as they moved through the microscope's field of view. There were also two instances where the movement of debris (probably resulting from organisms) created motility signals in the

brightfield motility output image. All of this makes the accurate enumeration of motile organisms in this type of sample more difficult. The differences in size between objects detected independently in both microscope modalities can also be observed in these data. This makes accurate size determination more difficult.

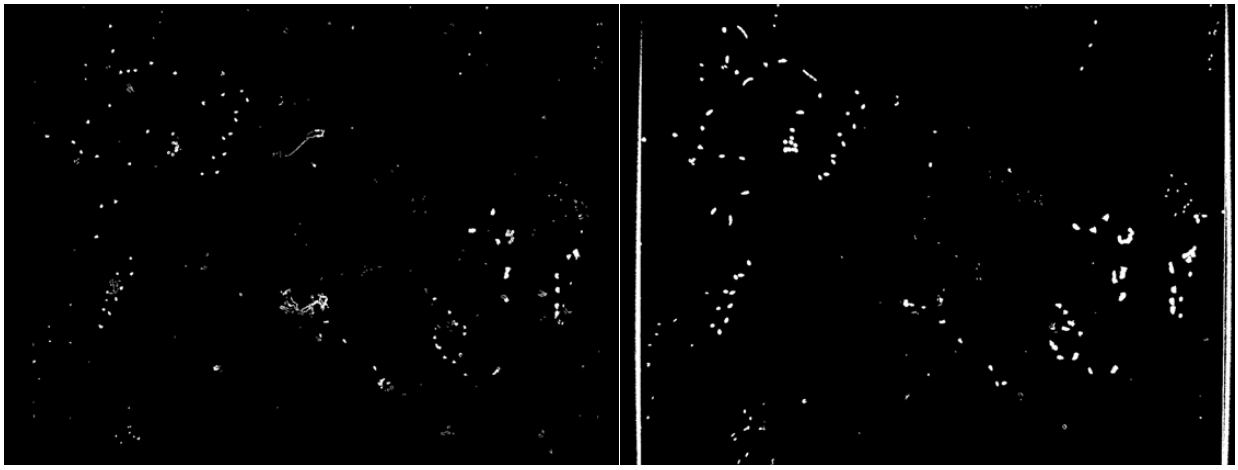

Figure B-7. Automated algorithm "motility" output images for the brightfield microscope modality (left) and for the epifluorescent microscope modality (on right). The complexity and number of organisms in this sample make the enumeration of motile organisms in these images difficult.

Figure B-8 shows the algorithm's composite output motility image. In this image, the paths of the organisms are better defined than in the individual microscope modality output motility images. However, the number of organisms in this sample makes interpreting these paths difficult. Very few of these organisms produced the 40 independent signals that could be produced if motile organisms produced the expected two signatures for each image pair analyzed. There are also signatures from several slow moving organisms (the signature becomes more like an elongated line rather than discrete object paths defined by points) as well as from debris that moved (likely induced by moving organisms) during the observation window. There is also a large variation in the size of the motile organisms detected in both microscope modalities.

A review of the data provided in Figures B-7 and B-8 show that with this more complex sample that accurately determining the viable organism count is not trivial based on an analysis of these output images. In this case, there are many possible interpretations for the number of organisms that created the motion paths shown in these images.

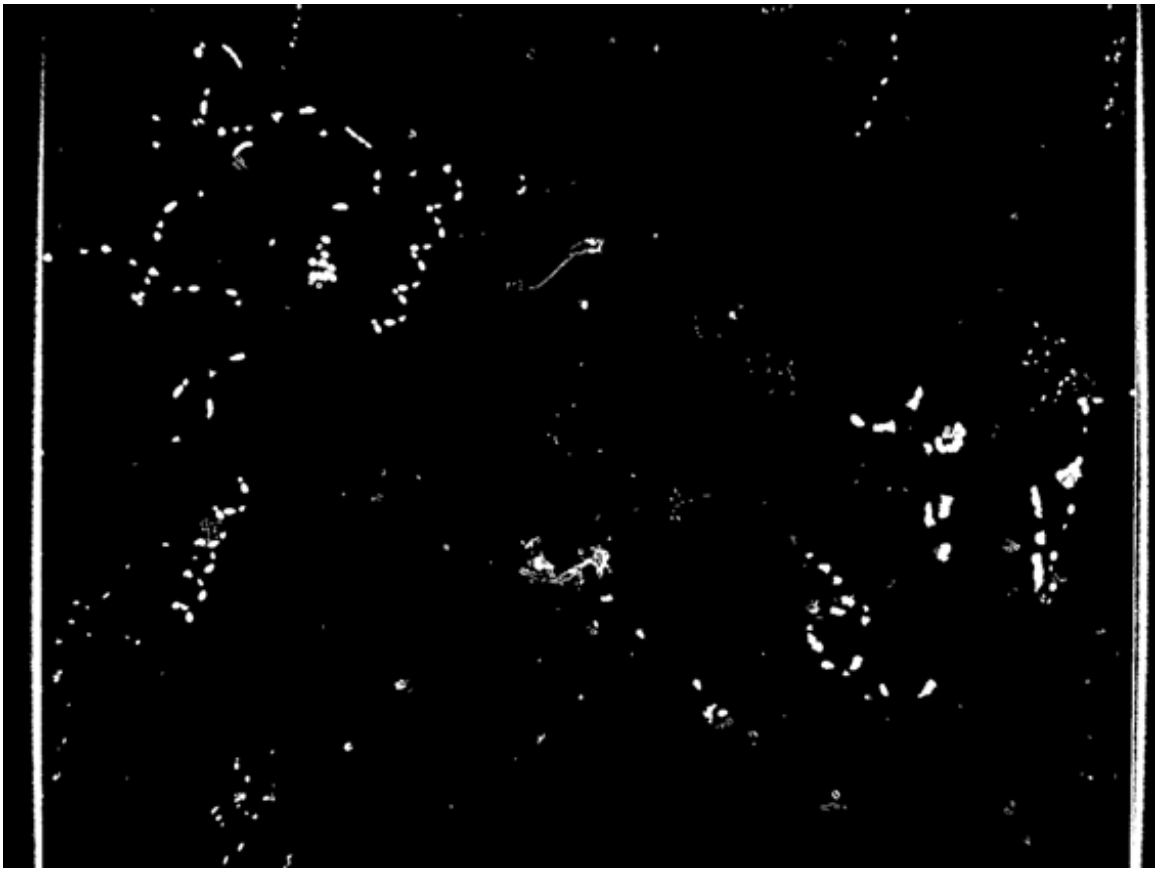

Figure B-8. Automated algorithm composite "motility" output image. The high organism concentrations in this sample make interpreting the motion paths more difficult. Also, note that most of the organisms detected in this image did not create 40 independent signals.

The data provided in this report section demonstrates some of the difficulties associated with developing accurate counts of motile organisms from the algorithm's output motility images. A more complex method than counting the number of objects and dividing by either 20 (individual microscope modalities) or by 40 (composite) is required for accurate motile organism enumeration. A more complex algorithm that develops a motile organism count but which also provides upper and lower bounds is under development. The upper and lower bounds will be developed by considering all of the possibilities for what might create motility signals (e.g., two "objects" can be from both the same object and two separate objects) in the output motility images at both the image pair and full observation window levels. The organism count will be generated by determining the most consistent data interpretation based on results obtained at both the image pair and full observation window levels as well as across both the individual and combined microscope modalities. It is believed that this type of approach will lead to a robust enumeration algorithm.

The data provided in this section also demonstrate some of the issues associated with accurately determining the size of detected organisms: the apparent size of organisms can be very different in the brightfield or epifluorescent microscope modalities. Although not as pronounced as with samples in the ≥ 10 µm to < 50 µm size class, microscope depth-of-focus issues combined with a 1 mm water column depth can also make it difficult to accurately determine the size of organisms in the ≥ 50 µm size class.

B.3 Status, Ongoing Activities, and Recommendations

As a result of this year's ongoing research and development activities, one major change was implemented in the Protocol for Automated Zooplankton Analysis. It is now recommended that unruled Sedgwick Rafter counting chambers with the microscope set-up to observe half of the slide area (equaling a 0.5 mL sample volume) be used to hold samples under the microscope when analyzing samples for organisms ≥ 50µm. The justification for making this change was provided in section B.1 of this Appendix.

During this year's ongoing activities, a significant number of complex samples were analyzed using automated analysis software. Because of these evaluations, the automation algorithms were significantly advanced during this program. There is an immediate need to develop and incorporate a robust enumeration algorithm for determining both the number of motile organisms ≥ 50 µm in size but also to generate upper and lower bounds for these counts. This requirement is driven by the complexity of the samples that will be analyzed using the automation algorithm. This has been described in section B.2 of this Appendix.

Work performed on complex samples has also shown that it is difficult to accurately determine the size of organisms that are detected in complex samples. For organisms in the ≥ 50 µm size class, the ability to accurately determine organism size is most impacted by the difference in the apparent size of objects that are observed in both the brightfield and epifluorescent microscope modalities. This has also been described in section B.2 of this Appendix.

As mentioned in the main body of this report, the Excel spreadsheet used for documenting data collection parameters and initial sample observations is currently under revision. It was felt that there would be no benefit in updating the report section that describes the Excel spreadsheet until after a new version is finalized. The modifications in this spreadsheet are being driven by the significantly greater amount of data that were generated in support of this program and are being made to help streamline the overall protocol while still ensuring that all pertinent information is accurately recorded prior to the collection of sample data.

It is also likely that some modifications will be made to the motility detection algorithms. As described in this appendix, the motility algorithms used with the epifluorescent image data use both an image normalization and adaptive thresholding method to generate binary images. It is believed that changing the threshold increment step used in this algorithm might improve its overall ability to detect motile organisms that are only weakly fluorescent. The motility algorithm used to analyze the brightfield image data currently uses image normalization and fixed thresholding methods. It is believed that the use of an adaptive thresholding algorithm in this algorithm will improve its ability to detect low contrast (compared to the debris) motile organisms in the brightfield imagery. It is recommended that ongoing incremental improvements continue to be made to the existing algorithms based on the analysis of additional complex sample data.

A critical next step will be to compare the results obtained using automated methods to manual counts generated on the same samples. As a result of the high number of samples that were generated and analyzed in support of this year's program, the data is currently in place to accomplish this once a more robust enumeration algorithm is developed. It is highly recommended that these comparisons be made as part of next year's program.

It is highly recommended that complex samples from other facilities and potentially other applications be generated and analyzed using the Protocols for Automated Zooplankton Analysis. It is also recommended that anytime a complex experiment is conducted at NRL, samples are generated and analyzed using the Protocols for Automated Zooplankton Analysis. The ability of the automated analysis algorithms to work well on data generated at other test facilities as well as from data generated from complex experiments conducted at NRL is important to the developed methods obtaining broad acceptance by the larger community involved with BWMS testing.